DRUGS EXPTL. CLIN. RES. **XXVIII**(5) 185-192 (2002)

SAFETY OF STABILIZED, ORALLY ABSORBABLE, REDUCED NICOTINAMIDE ADENINE DINUCLEOTIDE (NADH): A 26-WEEK ORAL TABLET ADMINISTRATION OF ENADA®/NADH FOR CHRONIC TOXICITY STUDY IN RATS

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Summary: *The safety of the stabilized, orally absorbable form of reduced nicotinamide adenine dinucleotide (NADH), known under the brand name ENADA®, was investigated over a period of 26 weeks. Eighty healthy rats (40 males and 40 females) were divided into two groups. One tablet ENADA®/NADH 5 mg per day was administered orally to one group while identical-looking white tablets not containing NADH (placebo) were given to the other group. The following parameters were statistically analyzed: body weight, body weight gain, food consumption, hematology, clinical chemistry, organ weight and organ histology. Clinical signs and mortality were recorded. There were no deaths associated with the study drug and no treatment-releated clinical signs. No differences in body weight between the placebo and the ENADA®-treated males were observed. In the second half of the treatment period (weeks 13-26) females treated with NADH gained significantly (p <0.05) more body weight than the controls. Food consumption in the treated males was similar to that in controls. From approximately week 15, the treated females consumed up to 10% more food than the controls. No differences were observed between the control and the treated groups in terms of hematology or clinical chemistry parameters. There was no apparent treatment-related effect on urine analysis parameters or on either the absolute or the relative organ weight. Furthermore, no macroscopic evidence of specific target organ toxicity associated with the test drug was observed. Histological findings in the treated rats were generally similar to those in control rats. A daily dose of 5 mg in a rat corresponds to a dose of 175 mg per day in a 70-kg human. This is 175 times the recommended daily dosage of 1 ENADA® tablet per day. Hence ENADA®/NADH 5 mg tablets can be generally regarded as safe.*

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Introduction

The objective of this study was to examine the safety and potential toxicity of ENADA®/NADH in rats

0378-6501/2002/5 00185+7 $02.50/0

receiving oral tablet administration for 26 weeks. ENADA® is the brand name for the stabilized, orally absorbable form of the reduced form of coenzyme nicotinamide adenine dinucleotide (NADH) (1, 2). This product has been used in several clinical trials for energy-deficient conditions such as chronic fatigue syndrome (3). A prerequisite for a study with human subjects was to demonstrate the safety of this drug in a chronic toxicity study. The oral route of administration was chosen because it is a human therapeutic route.

Materials and methods

Study drug

The study drug, ENADA®, consisted of white tablets containing 5 mg of NADH. The ENADA®/NADH 5 mg enteric-coated tablet has the following composition: active ingredient β-NADH (nicotinamide adenine dinucleotide – reduced form) 5 mg. Other ingredients are: i) (1) d-mannitol (Merck Co., Germany) 54.30 mg; ii) sodium bicarbonate (Merck Co.) 6.0 mg; iii) microcrystalline cellulose (Merck Co.) 1.36 mg; iv) magnesium stearate (Merck Co.) 1 mg; and v) sodium ascorbate (Merck Co.) 0.34 mg. The total tablet weight was 68.00 mg. Coating material was methacrylic acid copolymer (Eudragit®; Röhm GmbH, Germany). When not in use, the test article was stored at ambient temperature (10 °C to 30 °C) in darkness and was protected from moisture.

The control animals received white tablets with the same composition as the ENADA® tablets but without NADH.

Experimental design and dose levels

Table I shows the dose levels. The study drug was administered once daily for a minimum of 26 weeks, excluding the day of necropsy.

Test system

A sufficient number of rats of the Crl:CD(SD)BR strain was obtained from Charles River Ltd. (Margate, UK) to provide 40 healthy rats of each sex.

The rats were obtained as young adults in weight ranges of 333-359 g (males) and 232-255 g (females). All rats were given an external examination for signs of ill health on arrival. They were acclimatized to the study room for approximately 15 weeks, during which time their health status was reassessed and their suitability for experimental purposes confirmed. At the start of treatment, the males weighed 529–732.9 g and the females 309.3–401.7 g.

The rats were housed in a single, exclusive room, which was air-conditioned to provide a minimum of 15 air changes per hour. The room was routinely maintained at a temperature of 19-25 °C with a relative humidity of 40-70%.

Table I *Doses*

	Group	Dose level	Number of rats	
Group number	Description	(tablets/day)	Male	Female
1	Control	0.00	20	20
2	Treated	1	20	20

Fluorescent lighting was controlled automatically to give a 12-h of light/dark cycle (06:00-18:00 h).

The rats were housed five per cage, according to sex, in stainless steel mesh cages suspended over cardboard lined trays. The cardboard liners were replaced as often as was necessary to maintain hygienic conditions.

Throughout the study the rats had free access to SQC Rat and Mouse Maintenance Diet No. 1, Expanded, Ground Fine (Special Diets Services, Ltd., Witham, UK) except during the overnight fasts before laboratory investigations and necropsy.

Filtered drinking water was available *ad libitum* from an automatic watering system, except during urine collection, when the rats were deprived of water overnight.

Allocation to treatment group

The rats were assigned to treatment groups during the acclimatization period using a randomization procedure based on stratified body weight. The group mean body weights were calculated and inspected to ensure that there were no unacceptable differences between the groups. Treatment group positions were assigned using a set of random letter permutations.

Identification of the test system

After allocation to a treatment group, each rat was individually identified by electronic implant (Biomedic Data Systems, Inc., UK) (Table II). A color-coded card on each cage gave information including study number and rat number.

Table II *Rat identification*

		Rat identification numbers	
Group number	Color code	Male	Female
1	buff	1-20	41-60
2	pink	21-40	61-80

Experimental observations

All rats were examined daily for signs of ill health or overt toxicity. In addition, each rat was given a detailed clinical examination at weekly intervals. An individual record was maintained of the clinical condition of each rat. Additionally, all rats were observed at appropriate intervals after dosing. All rats were examined twice daily to detect any that were dead or moribund.

Individual body weights were recorded before treatment on the first day of the study, at weekly intervals thereafter and at necropsy. The amount of food consumed by each cage of rats was determined weekly.

The eyes of all rats were examined twice predose and in week 25. The examination was carried out using a Keeler indirect ophthalmoscope. A mydriatic agent (1% tropicamide) was instilled into the eyes before the examination.

Laboratory investigations

Blood samples were obtained from the 10 males and 10 females in each group with the highest identification numbers. Samples were collected before dosing and in weeks 4, 13 and 26.

The samples were collected by orbital sinus puncture under light halothane anesthesia following an overnight fast.

Urine samples were collected from the 10 males and 10 females with the highest identification numbers in weeks 4, 12 and 25. The samples were collected overnight from rats deprived of food and water.

Hematology. The following parameters were measured in blood collected into EDTA anticoagulant:

hemoglobin concentration, mean cell volume, red blood cell count and indices (including mean cell hemoglobin, mean cell hemoglobin concentration, packed cell volume), total and differential white blood cell and platelet count.

Further blood samples were collected into 3.8% trisodium citrate anticoagulant and prothrombin and activated partial thromboplastin times were measured.

Clinical chemistry. The following parameters were measured and plasma was separated from blood collected into lithium heparin anticoagulant: aspartate aminotransferase, alkaline phosphatase, sodium, chloride, inorganic phosphorus, urea, creatinine, albumin, total cholesterol, potassium, calcium, glucose, total bilirubin, total protein and the albumin/globulin ratio.

Urine analysis. The following parameters were determined: volume, specific gravity, protein, pH, glucose, bilirubin, ketones, blood, reducing substances, urobilinogen and microscopy of deposits.

Pathology

The following procedures were applied to all rats killed at the end of the study and, where possible, to the rat killed *in extremis*.

Necropsy. Rats were given an intraperitoneal injection of sodium pentobarbitone and, following exsanguination, a full internal and external examination was made under the general supervision of a pathologist. All lesions were recorded. Necropsies were performed in cage position order after an overnight period without food.

The necropsies were carried out on 2 consecutive days, with the males and the five females with the lowest identification numbers killed on one day and the remaining females on the following day.

Organ weights. The following organs were dissected free from fat and other contiguous tissue and weighed before fixation: adrenals, brain (including brain stem), heart, kidneys, liver, ovaries, pituitary, prostate, spleen, testes (with epididymides) and thyroids (with parathyroids). Left and right organs were weighed separately.

Histology. Samples of the following tissues were fixed in 10% neutral buffered formalin: adrenals, aorta, brain (including brain stem), caecum, colon, duodenum, eyes (with optic nerves), femur (including articular surface), harderian gland, heart, ileum, jejunum, kidneys, lachrymal glands, liver, lungs (with mainstem bronchi), lymph nodes (mandibular and mesenteric), mammary glands (females only), nasal turbinates, esophagus, all gross lesions, ovaries, pancreas, pituitary, prostate, rectum (with anus), salivary gland (submandibular), sciatic nerve, seminal vesicles, skeletal muscle (quadriceps), skin, spinal cord (lumbar, cervical, thoracic), spleen, sternum (with bone marrow), stomach, testes, thymus, thyroids (with parathyroids), tongue, trachea, urinary bladder, uterus (corpus and cervix), vagina and Zymbal's gland. The eyes and optic nerves were fixed in Davidson's Fluid and the bone marrow smears were fixed in methanol

Tissues specified above from all rats were embedded in paraffin wax BP, sectioned at a nominal thickness of 5 µm, stained with hematoxylin and eosin and evaluated by the study pathologist using light microscopy. Bone marrow smears from all rats were prepared at necropsy (fixed in methanol) but were not examined.

Statistical analysis

The following parameters were analyzed statistically: i) body weight: week 0 and day of necropsy; ii) body weight gain: weeks 0 to 13, 13 to 26 and 0 to

26; iii) food consumption: weeks 1 to 13, 14 to 26 and 1 to 26; (iv) hematology: before dose, weeks 4, 13 and 26; (v) clinical chemistry: before dose, weeks 4, 13 and 26 and; vi) organ weights adjusted to overall mean necropsy body weight.

The start of treatment body weights, necropsy body weights, all body weight gains, food consumption intervals and clinical chemistry and hematology variables were analyzed using Student's *t*-test. Levene's test for equality of variances between the groups was also performed (4). When this test gave evidence of heterogenous variances and an alternative analysis was deemed necessary, the data were analyzed using nonparametric methods. The nonparametric test used was the Wilcoxon rank-sum test (5). The hematology variable (neutrophils - females only) and the clinical chemistry variables (aspartate aminotransferase and alanine aminotransferase) were log-transformed before analysis because previous research into the large-scale distribution of these variables indicates that they have an approximately log-normal distribution.

All organ weights were analyzed using analysis of covariance (6) and Student's *t*-test (7), using the necropsy body weight as a covariate. This analysis depends on the assumption that the relationship between the organ weights and the covariate is the same for all groups; the validity of this assumption was tested.

Levene's test for equality of variances across the groups was also performed for all organ weights. If this showed evidence of heterogenous variances, where necessary, the data were analyzed using Student's *t*-test (7) for the absolute organ weights and the organ weight to necropsy body weight ratios. Levene's test was again performed on these data and when evidence of heterogenous variances was found, alternative analysis was performed.

Some variables were not analyzed because much of the data had the same value and/or there were few distinct values.

Results

Clinical signs

No treatment-related clinical signs were observed.

Mortality

One treated female (rat number 66) was killed in week 24 due to eye lesions. There was no evidence to suggest that the condition of the rat was associated with study drug toxicity. There were no other deaths during the treatment period.

Body weight

There were no treatment-related differences in body weight. From weeks 13 to 26, treated females gained significantly more body weight than did controls ($p < 0.05$). From weeks 0 to 26, treated females gained 34% more weight than the controls but this was not statistically significant.

Food consumption

The food consumption of treated males was similar to that of controls. From about week 15 the treated females consumed up to 19% more food than the controls and this was shown to be statistically significant ($p < 0.05$).

Ophthalmoscopy

During the examination in week 25, a treated female (rat number 69) showed slight bilateral lens opacity. This was a common finding and considered to be unrelated to treatment. There were no other ophthalmic events.

Laboratory investigations

Hematology. No treatment-related differences were found between the control and treated groups in the hematology parameters measured in weeks 4, 13 and 26.

Clinical chemistry. Minor variations in the clinical chemistry parameters measured in weeks 4, 13 and 26 were observed, but no clear treatment-related effect was noted.

Urine analysis. There was no apparent treatment-related effect on the parameters measured in samples collected in weeks 4, 12 and 25.

Organ weights

No apparent effect of treatment was found in either the absolute or the relative organ weights. The heart weights of the treated males were significantly lower ($p < 0.05$) than those of the controls. Individual heart weights were within the control range and the difference was small. This was therefore considered to be unrelated to treatment. The weight of the right adrenal for one control male (rat 5) was increased compared with the left adrenal weight due to a mass.

Necropsy

At the terminal necropsy, the majority of tissues were macroscopically unremarkable. The findings were generally consistent with the expected background in rats of this strain. There was no macroscopic evidence of specific target organ toxicity associated with administration of the study drug.

Histology

Histology findings in control rats were generally infrequent, minor and consistent with the expected background pathology in rats of this strain. Findings in treated rats were generally similar to those in controls, but there were minor intergroup variations in background histology findings, especially in females. In particular, more acyclic ovaries arrested in the follicular phase were seen in treated females than in control females. In the kidney, the incidence of cortico-medullary mineralization was lower and that of glomerulonephropathy was higher in treated females than in controls.

The incidence of both acyclic ovaries and glomerulonephropathy tends to increase with age in rats of this strain. Therefore, the aging process was possibly marginally affected in treated females. However, such minor intergroup variations in one sex could be a chance event.

There was no microscopic evidence of specific target organ toxicity associated with administration of the study drug.

Discussion

Oral administration of one NADH tablet containing 5 mg NADH for 26 weeks to Crl:CD(SD)BR rats was well tolerated.

No effect on males was observed. Female rats given NADH gained more weight and consumed more food during the last half of the study. The effect was small and could have been a chance event rather than a treatment-related effect. In addition, some minor variations in the pathological analysis were observed. In particular, a greater number of acyclic ovaries arrested in the follicular phase were seen in treated females than in control females. In the kidney, the incidence of cortico-medullary mineralization was lower and that of glomerulonephropathy was higher in treated females than in controls.

The incidence of both acyclic ovaries and glomerulonephropathy tends to increase with age in rats of this strain. Therefore, the aging process might have been marginally affected in treated females. However, such minor intergroup variations in one sex only could be a chance event. The toxicological significance of these findings is equivocal.

In conclusion, no treatment effects were observed in males. In females, a few changes of equivocal significance were noted, but there was no evidence of specific target organ toxicity.

The dose of 5 mg for a rat weighing about 400 g corresponds to a dose of 875 mg for a 70 kg (70,000 g) human subject. In terms of ENADA® tablets, that means 175 tablets of ENADA®/NADH 5 mg per day, given over a period of 26 weeks, show no evidence of toxicity. The recommended daily dose of ENADA® for healthy individuals is 1 5-mg ENADA® tablet per day. For the elderly or individuals with chronic fatigue, the most effective dose seems to be 4-6 ENADA® 5 mg, although a double-blind, placebo-controlled, clinical study showed that 82% of patients with chronic fatigue syndrome derived a benefit from as little as two 5-mg tablets of ENADA® mg per day after a 6-month treatment period.

Even with a daily dose of 5 tablets ENADA®/NADH 5 mg, the well-tolerated dose in rats is 35 times higher. In other words, a dose of 35 × 5 ENADA® tablets per day for 6 months will produce no toxicity.

Based on these findings, ENADA®/NADH can be generally regarded as safe. More than a million consumers of ENADA® who have been regularly taking this product for a couple of years have reported no side effects and may provide additional evidence for the safety of ENADA®.

Possibly, a toxicological effect has not been observed because the NADH in the tablets is not absorbed in the gastrointestinal tract. This argument can be disproved by the results of three studies. One study showed that NADH passes the intestinal mucosa undegraded by passive diffusion (8). In another study performed at Georgetown University, spontaneous hypertensive rats received one ENADA®/NADH 5-mg tablet per day for 3 months. This treatment significantly decreased blood pressure, lipid peroxidation, total cholesterol and low-density lipoprotein cholesterol (9). Since this was a placebo-controlled study and niacinamide was used as placebo, the treatment effect was evoked by NADH, implying that NADH was absorbed and became bioavailable NADH.

The bioavailability of NADH after oral application was proven by measuring the NADH concentration in the brain cortex using laser-induced fluorescence. Twenty minutes after oral application of one 5-mg NADH tablet, NADH-specific fluorescence increased (10). This observation provides evidence that NADH is absorbed and can pass the blood-brain barrier and thus supply the central nervous system with greater NADH and energy.

A further argument for the safety of NADH comes from a study in dogs. The maximum tolerated dose was found to be 500 mg per kg body weight after intravenous application. Even under these conditions of an extremely high NADH concentration in the blood, no toxicological effects were observed (11).

Acknowledgment

This study was performed at the Laboratory of Corning Hazleton Europe in Harrogate, England, under

the supervision of the study directors J.F. Richards and H. Nutall. It was performed in accordance with the UK Principles of Good Laboratory Practice. Their very professional work is gratefully acknowledged.

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