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On the Safety of Reduced Nicotinamide Adenine Dinucleotide (NADH)

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The objective of the study was to determine both the toxicity of the stabilized orally absorbable form of nicotinamide adenine dinucleotide (NADH) (ENADA®) and the maximum tolerated intravenous dose (MTD) of βNADH (the reduced form of NADH) in beagle dogs. The administration of the stabilized orally absorbable form of NADH to beagle dogs at dose levels of 20, 100, and 150 mg/kg for 14 days elicited no signs of a toxicological effect. A transitory change in stool formation was observed with the intermediate and high dose in males. There were also apparent increases in adrenal, heart, kidney, liver, brain, and thyroid weights, particularly in males, but none of these changes were considered to be toxicologically significant. In addition, four dogs (two of each sex) received intravenous infusions of 100 mg NADH/kg/day for 4 days, followed by 200 mg NADH/kg/day for 3 days, followed by 500 mg NADH/kg/day for 4 days, and 1000 mg NADH/kg/day on the final day. At the end of the MTD phase, the control animals that had received saline solution in the MTD phase were used to evaluate the potential toxicity of the established MTD. These animals received 500 mg NADH/kg/day for 14 days (fixed dose phase). There were no deaths. At dose levels between 100 and 1000 mg/kg/day, effects on the cardiovascular system and also some evidence of an effect on the central nervous system and on the adrenals were observed. At doses of 500 mg/kg/day and above, food consumption and body weight were reduced. On the basis of the observed changes, the maximum intravenous dose of NADH tolerated by beagle dogs was considered to be 500 mg/kg/day. There were no gross histological findings indicative of toxicity in the organs of tissues examined. Based on these findings, the stabilized orally absorbable form of NADH (ENADA) can be regarded as safe.

KEY WORDS: NADH, toxicity studies, maximum tolerated dose (MTD), orally absorbable NADH

Introduction

Nicotinamide adenine dinucleotide hydride (NADH) (Oriental Yeast, Tokyo, Japan) is a coenzyme essential for energy production, hence present in all living cells. This coenzyme has been used as a new therapeutic approach for Parkinson's disease, for depression, and for chronic fatigue syndrome.[1-3] It is known that toxicology studies are a prerequisite for more extensive, multicenter clinical trials to be approved by health authorities in the European Union as well as in the US. Therefore, the toxicity of the stabilized orally absorbable form of NADH (ENADA®) was determined following oral administration in beagle dogs for 14 days at three dose levels (range: 20–150 mg/kg). In humans, therapeutic effects have been reported for the stabilized orally absorbable form of NADH at dose levels of 10 mg/day.[3]

A maximum tolerated intravenous dose (MTD) of NADH was assessed in beagle dogs, and the toxicity of this dose level was evaluated for 14 days. The intravenous route of administration was chosen as a possible human therapeutic route. The canine species was selected for these toxicity studies because it is one of the nonrodent species recommended by various regulatory authorities.

Materials and Methods

Oral Application

Experimental Design

Drugs. NADH was supplied as white tablets, containing 5 mg of NADH each (ENADA® tablets containing 5 mg βNADH, 2.92 mg sodium bicarbonate, 0.3 mg sodium ascorbate, 49.55 mg mannitol, 0.9 mg magnesium stearate, 7.1 mg methacrylate [Eudragite®, Röhm GmbH, Darmstadt, Germany], and 1.18 mg microcrystalline cellulose). All compounds except NADH and Eudragite® were from Merck A.G., Darmstadt, Germany.

Animals. For the subchronic toxicity study, 12 male and 12 female pure-bred beagles (Hazleton Research Products, Pennsylvania) were used. Before delivery, they received a course of treatment at the supplier's premises for endo-parasites and were vaccinated against common canine pathogens. Shortly after arrival, the beagles were revaccinated and received oral antihelminthic treatment. The animals were assigned arbitrarily to treatment groups during the acclimatization period using a randomization procedure based on stratified body weight. Each beagle was individually identified by a subcutaneous electronic implant. A color-coded card on the kennel reported on study and animal number. Daily throughout this investigation, each beagle was offered 400 g of SQC Laboratory Diet A, Expanded (Special Diets Services, Ltd., Witham, Essex, UK). Any uneaten diet was removed and weighed each afternoon then discarded. Filtered drinking water was available *ad libitum*. The beagles were housed in four rooms, singly during the working day and where possible in groups of three of the same sex and group overnight. The rooms were air-conditioned (16–22 °C; relative humidity: 40–80%) and fluorescent lighting controlled (12-h light: 7 AM–7 PM). At the start of treatment, males weighed 8.65–10.95 kg and females 7.05–9.90 kg (age: 7–9 months). Dose levels of 0, 20, 100, and 150 mg/kg/day were chosen. Gelatine capsules were used as vehicle. Doses to the nearest 5 mg (whole tablet) were administered. Control animals received empty capsules. The required number of tablets for each individual were counted, then placed into the minimum number of capsules practicable (approximately 30 tablets per capsule) and assigned to the beagle by placing in a labeled pot. The low dose was a multiple of the intended therapeutic dose. Individual doses were adjusted weekly according to the latest body weight. The capsules were administered twice daily for a minimum of 14 days, excluding the day of the sacrifice.

Observations at Oral Subchronic Toxicity Experiments

All beagles were observed in the morning, before feeding, and again in the afternoon. In addition, all

beagles were given a detailed clinical examination at weekly intervals. Individual food consumption was determined daily and body weights were recorded weekly during the study and before sacrifice. The eyes of all beagles were examined at baseline and in week 2 (mydriatic agent: 1% tropicamide, Keeler indirect ophthalmoscope). Electrocardiographic (ECG) recordings (leads I, II, III, aVR, aVL, and aVF) were taken from all beagles at baseline and in week 2, 3 hours after the afternoon dose. The heart rates and PR, QRS, QT, and QTc intervals were determined on lead II (automated ECG recorder). Blood pressure recordings (diastolic, systolic, and mean arterial) were made on all beagles before the morning dose and 3 hours after the afternoon dose in week 2. Direct measurements were made from the ear artery.

Laboratory Investigations

Blood samples were collected from the jugular vein from all beagles at baseline and at the end of week 2 before dosing (after overnight period without food). For hematological investigations EDTA-blood was collected and the following parameters were measured: hemoglobin concentration, hematocrit, red blood cell count and hematological indices (MCV, MCH, and MCHC), and total and differential white blood cell and platelet count. Further blood samples were collected on 3.13% trisodium citrate anticoagulant and prothrombin and activated partial thromboplastin times were measured.[4] For clinical chemistry measurements blood was collected on lithium heparin anticoagulant, and the following parameters were measured: glucose, urea, creatinine, total bilirubin, total protein, albumin/globulin ratio, calcium, potassium, total cholesterol, aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and gamma glutamyl transferase.[5-14]

Pathology

The beagles were killed by intravenous injection of sodium thiopentone and exsanguination. A full external and internal examination was made and all gross findings were recorded.

Organ weights. The following organs were dissected free from fat and other contiguous tissue and weighed before fixation: adrenals, brain (including brain stern), heart, kidneys, liver, lungs, ovaries, pancreas, pituitary, prostate, spleen, testes and epididymides, thymus, thyroids, and uterus.

For histological investigations the following tissue samples were fixed in 10% neutral buffered formalin (the eyes and optic nerves were fixed in Davidson's fluid): adrenals, aorta, brain (including brain stern), caecum, whole colon, epididymides, eyes (with optic nerves), femur, gall bladder, heart, ileum, injection sites, jejunum, kidneys, lachrymal glands, liver, lungs (with mainstem bronchi), lymph nodes (mandibular and mesenteric), esophagus, ovaries, all gross lesions, pancreas, pituitary, prostate, rectum (with anus), salivary gland (submandibular), sciatic nerves, skeletal muscle (quadriceps), skin and mammary gland, spinal cord (lumbar, cervical, thoracic), spleen, sternum (with bone marrow).

Intravenous Application

Experimental Design and Dose Levels

Drugs. NADH was dissolved in physiological saline and administered intravenously at a rate of 0.5 mL/minute into the cephalic vein. Four male and four female pure-bred beagles (Hazleton Research Products, Pennsylvania) were used for the intravenous maximum tolerated dose (MTD) and fixed dose (FD) intravenous toxicity study. The beagles were held in stock for about 3 weeks before the start of the MTD phase (for treatment at the supplier's premises, see oral NADH application experiments). At the start of the experimental phase the male animals weighed 7.85–12.30 kg and the females 8.20–9.65 kg (age: 9–11 mo). For the MTD phase, two groups (2 males and 2 females per group) received intravenously either the physiological saline for 14 days (control group) or NADH solutions of varying doses at different times during the investigation (Fig. 1). Upon completion

of the MTD phase, the control beagles, which had received physiological saline in the MTD phase, were assigned to an FD phase and treated with 500 mg NADH/kg/day (the dose was based on the results from the MTD phase). A constant dose volume of 5 mL/kg was used for both the MTD and FD phases of the study. The required volume was adjusted daily during the MTD phase and twice weekly during the FD phase, calculated from the recorded individual body weight. During the MTD phase, NADH was administered once daily, excluding day 13. During the FD phase, NADH was administered once daily for a minimum of 14 days. The infusion solutions of NADH were prepared daily prior to application for each group. The beagles were housed in a single room, singly during the working day and in pairs of the same sex and group overnight.

The environment, diet and water supply, the assignment to treatment groups and identification were in accordance with the conditions described for the oral application experiments.

Experimental Observations on MTD and FD Phases

All beagles were observed in the morning, before feeding, and again in the afternoon. Clinical examinations were performed at weekly intervals. Additional observations were made particularly between 2 and 4 hours after dosing, when the dose level was increased (MTD phase), and on day 1 of the FD phase also between 8 and 12 hours post dosing. Individual food consumption was determined daily throughout the study. Body weights were recorded daily during the MTD phase, twice weekly during the FD phase, and before necropsy. Blood pressure recordings were made (as in the oral experiment) from all beagles before dosing, 10 minutes, and 1, 2, and 4 hours after dosing on day 1 of the FD phase. Blood pressure was measured by cannulation of the ear artery. On day 1 of the FD phase, the heart rate of each beagle was determined from the blood pressure tracings.

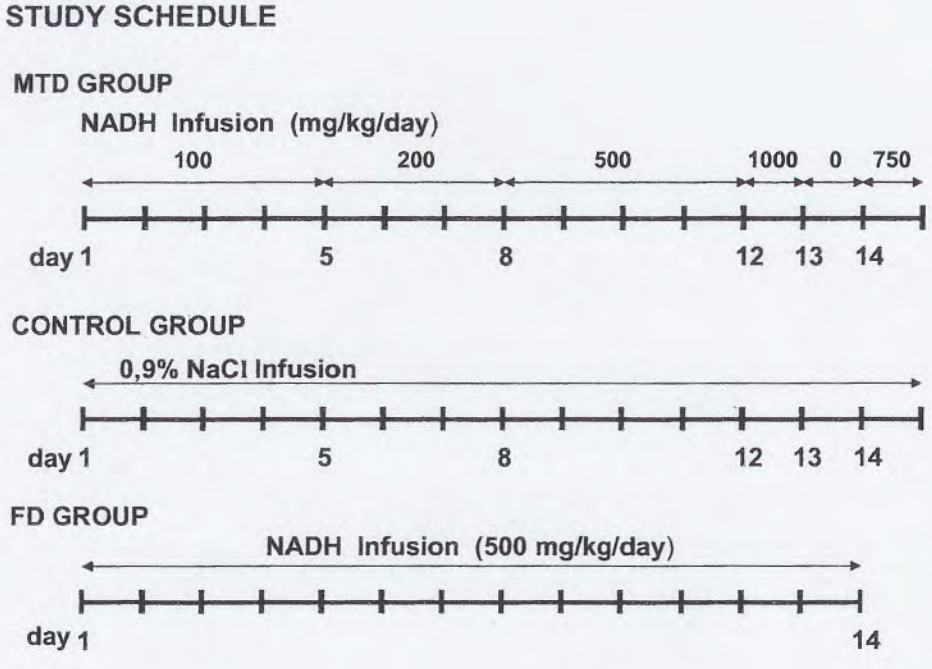

FIGURE 1. Study schedule for the MTD phase and FD phase experiments.

Laboratory Investigations

Laboratory and pathology investigations were performed identically to the ones described for the oral application of reduced NADH. Blood samples were collected from the jugular vein predosing and at the end of both the MTD and FD phases. Samples taken during the treatment periods were collected before daily dosing.

Statistical Evaluation

Mean values and standard deviations are given. Body weights, food consumption intervals, clinical chemistry, and hematology variables were analyzed using two-way analysis of variance (ANOVA).[16] Pairwise comparisons, separately for each sex, were made using the Dunnett's test, apart from the predose variables, for which pairwise comparisons are given. We used a protected t test (overall ANOVA: $p < 0.05$).[16,17] For each variable measured and for each sex separately, a regression test was performed to determine whether there was a linear relationship between increasing dose and response.[18] In the case of a significant result ($p < 0.05$) and if any of the pairwise comparisons were significant, a precedence was given to the pairwise tests, and the regression result was not reported. Levene's test for equality of variances across groups, between sexes, and for any interaction was also performed.[19] If these tests showed evidence of group effects or a sex-group interaction ($p < 0.02$), the Kruskal–Wallis, ANOVA, and Terpstra–Jonckheere test for dose response were used.[20-21] For predosing variables, only the ANOVA was used. If the Levene's test showed evidence of differing variances between the sexes only ($p < 0.02$), then a one-way ANOVA regression test and the Dunnett's test were performed for each sex separately as considered necessary. For predosing variables, the ANOVA and protected t test were performed. Regarding hematology, the basophil counts were not analyzed because more than 30% of the data points had the same value. The percentage of neutrophils, lymphocytes, monocytes, and eosinophil counts were analyzed in addition to their absolute values. All organ weights were analyzed by the covariance (ANCOVA) and Dunnett's tests, and the necropsy body weight as a covariate.[17,18] The Levene's test for equality of variances across the groups was also performed for all organ weights, and in all cases gave no evidence of heterogeneous variances. For the MTD and FD phase experiments the results were assessed using concurrent and historical control beagle data.

Results

Oral Administration

Clinical Observations and Hemodynamics

All beagles survived to their scheduled termination of the experiment. Treated and control beagles showed little change in body weight during the treatment period. The mean food consumption of treated males was similar to the controls, whereas in female low, intermediate, and high dose groups it was up to 23%, 27%, and 22% lower than that of controls. However, similar intergroup variations were apparent pretreatment. On days 3–7, all high-dose males passed loose or liquid feces. This occurred less frequently in week 2. Occasionally the feces of intermediate-dose males were loose, whereas the feces passed by control and low-dose beagles appeared normal. Females occasionally passed loose feces, but the incidence showed no relationship to the dose. No other clinical observations attributable to treatment were observed. Ophthalmoscopic examination revealed no treatment-related ocular changes.

Figures 2a and 2b show the mean arterial blood pressure and heart rate before and after 3 hours of oral administration of NADH in the different dosage groups. Three hours after dosing, there was no significant difference in the mean systolic blood pressure and heart rate in all groups. In addition, the electrocardiography tracings did not reveal any changes associable with the oral administration.

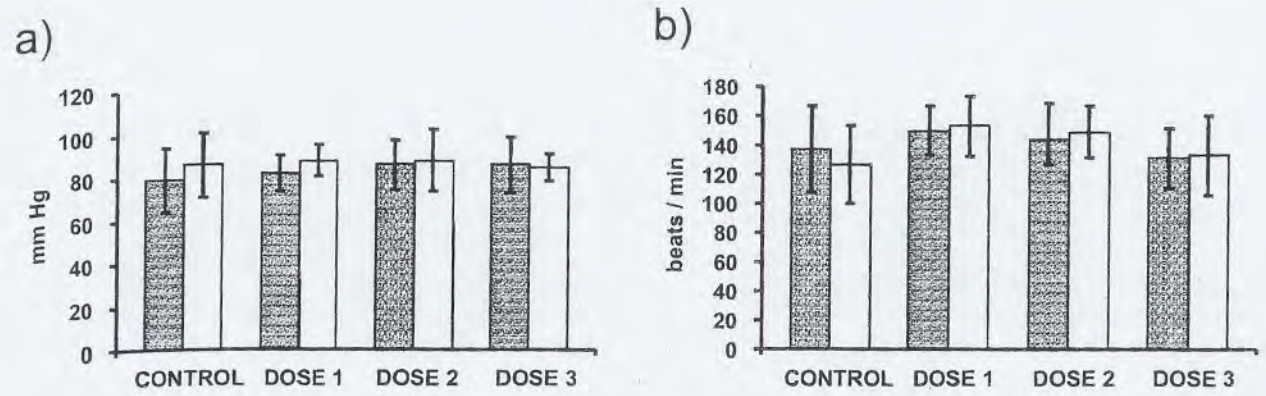

FIGURE 2. Mean arterial blood pressure (a) and heart rate (b) before and after 3h of oral administration of NADH in the different dosage groups (mean ± SD; $n = 6$). Dose levels: dose 1: 20 mg/kg; dose 2: 100 mg/kg; dose 3: 150 mg/kg.

Laboratory Investigations

Table 1 summarizes the hematological (a) and clinical chemistry (b) data obtained from controls and treated animals 2 weeks after oral administration of stabilized NADH. There were no significant changes in any of the hematological parameters in all dosage groups. The clinical chemistry parameters glucose, urea, creatinine, bilirubin, protein, and cholesterol did not differentiate between control and treated groups. In addition, no differences were seen in the plasma electrolytes between the groups. Figure 3 compares the values for aspartate aminotransferase (a), alanine aminotransferase (b), gamma glutamyl transferase (c), and alkaline phosphatase (d) before and 2 weeks after treatment. Intergroup differences within these clinical chemistry parameters were generally apparent before the start of treatment and were, therefore, considered unrelated to the oral NADH application. Only serum gamma glutamyl transferase activity increased to a high extent during the 2 weeks of the experiment in all treatment groups as well as in the controls. The calculations on a percentage basis revealed that the differences between groups in serum gamma glutamyl transferase activity were not related to treatment. In addition, the urine analysis after 2 weeks of oral application revealed an unchanged composition.

Pathology

The male animals showed a small but dose-related increase in adjusted mean liver weight. The means were 7%, 8%, and 12% higher than the control mean for low-, intermediate-, and high-dose males, respectively. The adjusted mean liver weight of the high-dose females was 5% higher than the control mean.

The adjusted mean brain weights for low-dose males and intermediate-dose females were 5% higher, and the means for high-dose males and females were 7% and 9% higher, respectively, than the control means. The adjusted mean adrenal weights of all treated male groups were 11%, 8%, and 21% higher than the control mean, and the adjusted mean thyroid weight of the high-dose males was 38% higher than the control mean. The adjusted combined mean kidney weights of the intermediate- and high-dose males were 7% and 6% higher, respectively, than the control mean. The weights of all other organs were unaffected by treatment.

Most tissues and organs were unremarkable on gross examination. Certain findings, such as red foci in lung, urinary bladder, stomach, cecum, and colon, occurred in treated groups compared to a zero incidence in controls. In general, these were isolated occurrences and were considered to represent ago-

TABLE 1. Hematological and Clinical Chemistry Parameters After Oral NADH Application (mean ± SD, $n = 6$)

a)

Hematology	Hb	Erythrocytes	Platelets	PT	APTT	Leucocytes	Lymphocytes
Week 2	g/dL	$10^6/cm^3$	$1000/cm^3$	s	s	$1000/cm^3$	$1000/cm^3$
Control	14.88 ± 0.74	6.72 ± 0.39	314 ±108	7.27 ± 0.26	11.03 ± 0.89	12.47 ± 1.35	4.18 ± 0.71
DOSE 1	15.83 ± 0.18	7.18 ± 0.14	272 ± 52	7.30 ± 0.30	11.80 ± 0.91	12.00 ± 1.96	4.07 ± 0.98
DOSE 2	14.97 ± 0.92	6.73 ± 0.54	308 ± 64	6.95 ± 0.84	11.08 ± 0.51	11.18 ± 1.26	3.08 ± 0.93
DOSE 3	14.87 ± 1.07	6.67 ± 0.48	327 ± 53	7.22 ± 0.57	11.40 ± 0.36	11.97 ± 1.85	3.33 ± 1.04

b)

Clinical Chemistry	Glucose	Urea	Bilirubin	Creatinine	Protein	Cholesterol
Week 2	mmol/L	mmol/L	µmol/L	µmol/L	g/L	mmol/L
Control	5.67 ± 0.31	5.08 ± 0.77	1.42 ± 0.46	74.33 ± 6.85	56.50 ± 1.61	2.62 ± 0.30
DOSE 1	5.80 ± 0.42	5.18 ± 0.47	1.52 ± 0.11	80.17 ± 7.24	59.17 ± 1.95	3.12 ± 0.09
DOSE 2	5.35 ± 0.30	5.23 ± 0.69	1.73 ± 0.39	74.50 ±11.42	56.00 ± 2.38	2.58 ± 0.49
DOSE 3	5.60 ± 0.33	5.30 ± 0.61	1.35 ± 0.10	73.33 ± 5.65	57.67 ± 2.87	2.93 ± 0.45

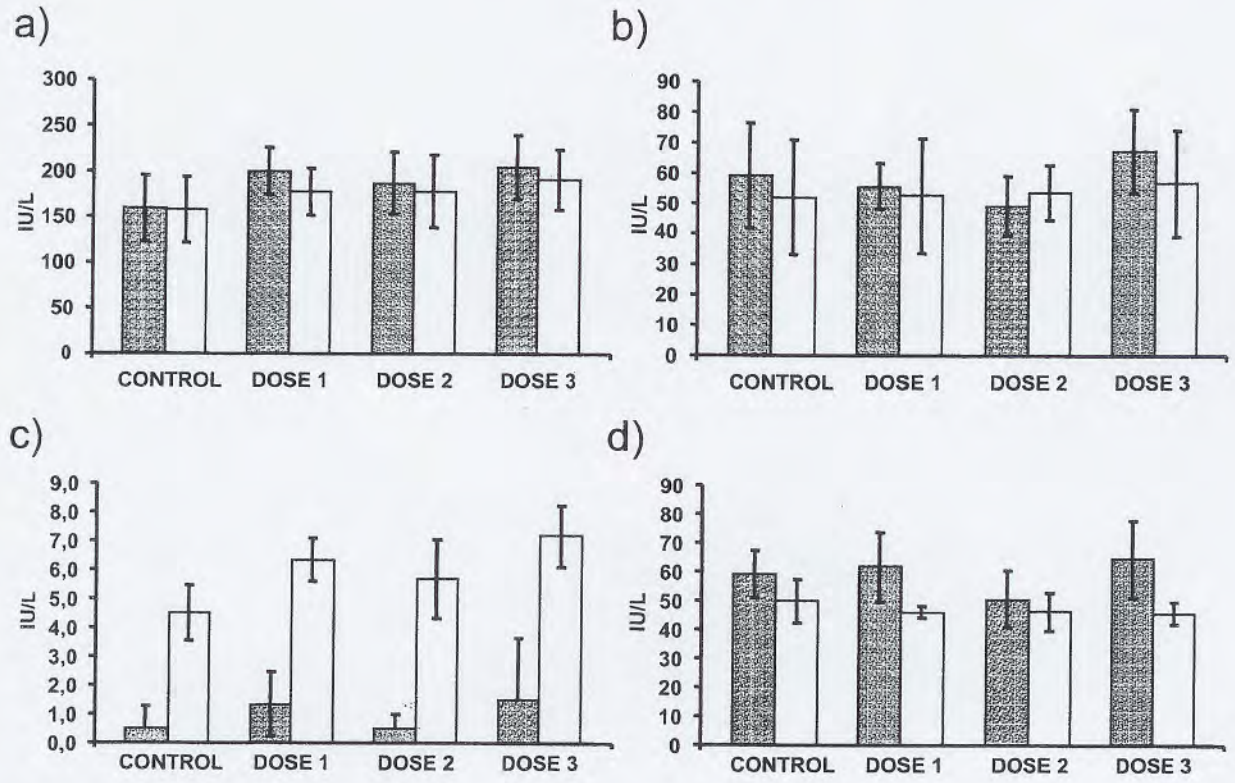

FIGURE 3. Aspartate aminotransferase (a), alanine aminotransferase (b), gamma glutamyl transferase (c), and alkaline phosphatase (d) values before and after 2 weeks of oral administration of NADH in the different dosage groups (mean ± SD; $n = 6$). Dose levels: dose 1: 20 mg/kg; dose 2: 100 mg/kg; dose 3: 150 mg/kg.

nal changes. There were no findings suggestive of treatment-related toxicity.

Histopathological findings were infrequent and comparable between control and treated groups (spectrum of young healthy dogs). Urinary cystitis occurred in some treated females compared to a zero incidence in controls, and there was no evidence of a dose-related increase in the incidence or severity. There were no further findings of an unusual nature or incidence in the organs or tissues examined.

Intravenous Application

Clinical Observations and Hemodynamics

Maximum tolerated dose. During infusion at all dose levels of the MTD experiments the treated beagles frequently became subdued, and the gums often became pale. At dose levels of 200 mg/kg/day and greater, the beagles frequently had tremors immediately after dosing and occasionally during dosing. In beagles receiving 500 mg/kg/day and greater, the pads of the feet became pale after dosing from day 10 onwards and were cold to touch. During dosing at 750 and 1000 mg/kg/day, the beagles often had warm ears and a dry nose. After dosing, the ears were cold and the beagles were described as being restless and having an arched back or hunched posture. The respiratory rate of one female during dosing at both levels and the respiratory rate of both males increased after dosing at 1000 mg/kg/day. However, after dosing at 750 mg/kg/day, this was exhibited by only one animal. The beagles also had an awkward gait, ataxia, and were unsteady on their feet after dosing at these levels. All treated beagles vomited

during the MTD phase, but the incidence showed no clear relationship with dose. Other signs exhibited by treated beagles included salivation, retching, lip licking, soft or mucoid feces which were often pale or yellow, red eyes, and the appearance of the third eyelid during dosing.

Fixed dose. The clinical signs observed during the FD phase were similar to those seen during the MTD phase. Treated beagles became subdued and had pale gums, cold (and less frequently warm) ears, blood-shot eyes, dry nose, and increased respiratory rate during dose administration. After dosing, all beagles had tremors and the pads of their feet were cold. All beagles vocalized and occasionally appeared agitated during dosing. Other signs seen included salivation, lip licking, red eyes, awkward gait, increased heart rate, panting, and the appearance of the third eyelid during dosing. Only one female vomited during the FD phase.

Body Weight

Maximum tolerated dose. At doses of 500 mg/kg/day and greater the body weight decreased. On day 13, the day after the administration of 1000 mg/kg/day, the body weights of the males were 4% and 6% lower, and the weights of the females were 6% and 8% lower than before treatment. The body weights of all controls on day 14 were within 2% of their weights on day 1.

Fixed dose. On day 14 of the FD phase the weight of animals (females) were slightly lower than on day 1 (3% and 6% in female and 2% in the male dogs, respectively).

Food Consumption

Maximum tolerated dose. At dose levels of 500 mg/kg/day and greater the food consumption tended to be slightly lower compared with the predose and control values. Neither of the animals ate more than 210–260 g/day during treatment at these levels (pretreatment they ate up to 370–400 g/day). At lower dose levels, the food intake was similar to that pretreatment.

Fixed dose. Food consumption was generally lower than predose (in week 2 as low as 250 g/day compared to 370–400 g/day). The only occasion during treatment when consumption approached similar levels was when the food was left available to the beagles overnight to encourage eating.

Cardiovascular changes. Figures 4a and 4b show the time course of the mean arterial blood pressure (MABP) and heart rate during the initial 3 hours of the FD phase on day 1. The mean arterial (systolic) blood pressure was lower 60 minutes after dosing but returned to the initial values after 120 minutes and remained at this level until 360 minutes. The drop in the MABP is consistent with the compensatory initial increase in heart rate after 10 minutes of NADH infusion on day 1 of the FD phase (Fig. 4b). However, after the following decrease in heart rate at 60 minutes, heart rates remained higher at all time points when compared with the predose rate.

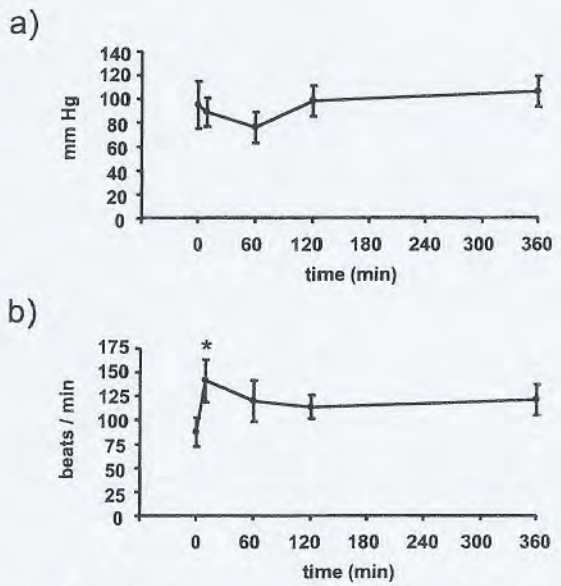

FIGURE 4. Mean arterial blood pressure **(a)** and heart rate **(b)** during the initial 3 hours of the FD phase on day 1 (mean ± SD; $n = 4$).*$p < 0.05$ versus predose.

Laboratory Investigations

Maximum tolerated dose. Table 2 compares the hematological (a) and clinical chemistry (b) data obtained on day 15 with the appropriate controls. There were no significant alterations. Only the serum cholesterol (Table 2b) and the alkaline phosphatase activity were significantly elevated on day 15 (Fig. 3d). However, despite the high doses of 1000 mg/kg/day on day 12 and 750 mg/kg/day on day 14, only the alkaline phosphatase showed a pathological value. Slight elevations were seen in urea and red blood cell count. The serum creatinine concentrations were also high compared with pretreatment and control values but did not reach significance. Other changes were generally also evident in the controls.

Fixed dose. Table 3 compares the hematological (a) and clinical chemistry (b) data obtained on day 14 of the FD phase with the predosing control values (controls: MTD study day 15). Figure 5 compares the values for serum aspartate aminotransferase (a), alanine aminotransferase (b), gamma glutamyl transferase (c), and alkaline phosphatase (d) activities in the MTD phase experiments with the appropriate controls and FD phase experiments with the predosing control values. On day 14 of the FD phase (termination of the experiment), only the serum cholesterol was significantly elevated compared to predosing. The mean 1.5-fold increase in the concentration of cholesterol was not sufficient to be considered as a pathological value. The other parameters were not affected by treatment.

On day 14 of the FD phase the plasma calcium concentration in all beagles was high compared with predose and day 15 of the MTD phase values. In contrast to the MTD phase experiments the activity of alkaline phosphatase was not elevated on

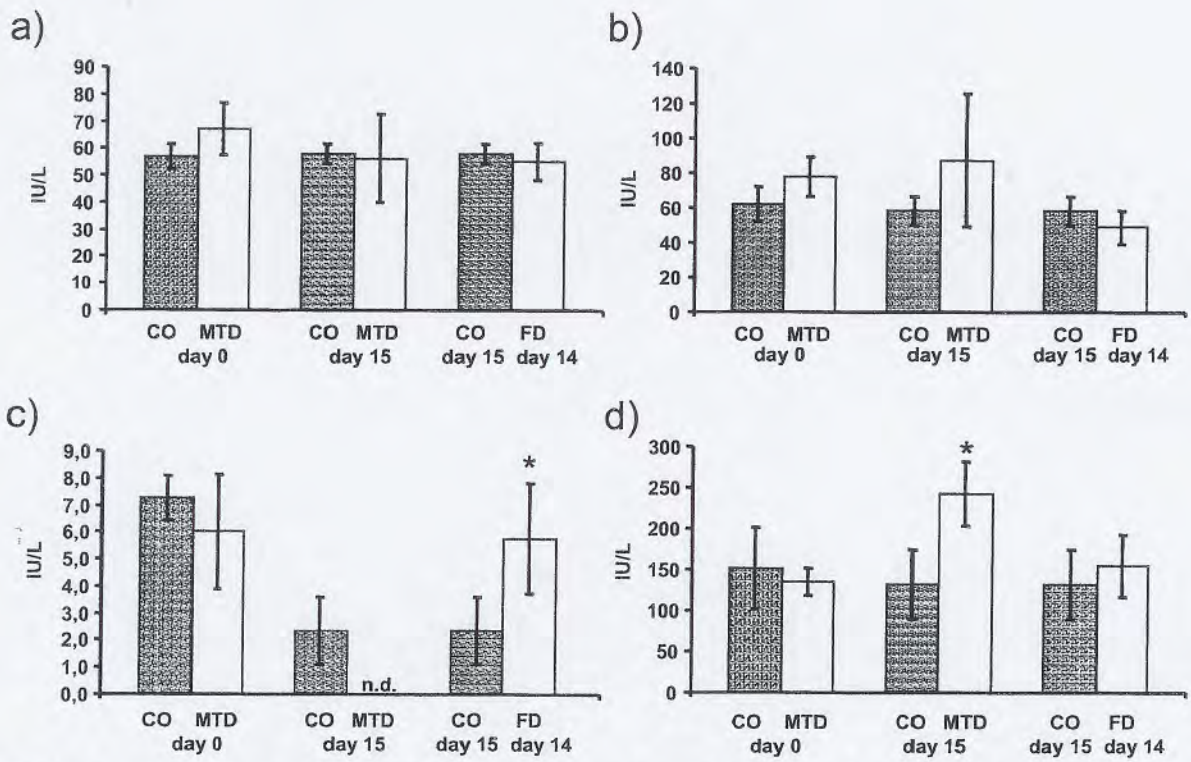

FIGURE 5. Aspartate aminotransferase (a), alanine aminotransferase (b), gamma glutamyl transferase (c), and alkaline phosphatase (d) values of the the MTD phase and FD phase experiments (mean ± SD; $n = 4$). *$p < 0.05$ versus control (CO) or predose in the FD phase experiments (CO, day 15).

TABLE 2. Hematological and Clinical Chemistry Parameters After Intravenous NADH Application—MTD Phase Experiments (mean ± SD, $n = 4$)

a)

Hematology	Hb	Erythrocytes	Platelets	PT	APTT	Leucocytes	Lymphocytes
day 15	g/dL	10^6/cm^3	1000/cm^3	s	s	1000/cm^3	1000/cm^3
Control	15.43 ± 1.20	6.71 ± 0.41	325 ± 59	9.73 ± 2.95	12.80 ± 1.06	10.20 ± 1.59	2.15 ± 0,48
MTD	17.23 ± 0.83	7.20 ± 0.31	272 ± 42	9.58 ± 2.64	12.08 ± 0.43	11.15 ± 1.87	1.80 ± 0.32

b)

Clinical Chemistry	Glucose	Urea	Bilirubin	Creatinine	Protein	Cholesterol
day 15	mmol/L	mmol/L	µmol/L	µmol/L	g/L	mmol/L
Control	5.30 ± 0.16	5.98 ± 0.59	2.93 ± 0,72	79.00 ± 7.18	56.00 ± 2.34	2.95 ± 0,72
MTD	5.18 ± 0.48	6.63 ± 0.74	3.93 ± 1,29	96.50 ± 19.05	58.50 ± 2.96	5.55 ± 0.97 *

*$p < 0.01$

TABLE 3. Hematological and Clinical Chemistry Parameters After Intravenous NADH Application—FD Phase Experiments (mean ± SD, $n = 6$)

a)

Hematology	Hb	Erythrocytes	Platelets	PT	APTT	Leucocytes	Lymphocytes
	g/dL	$10^6/cm^3$	$1000/cm^3$	s	s	$1000/cm^3$	$1000/cm^3$
Control (pre-dose)	15.43 ± 1.20	6.71 ± 0.41	325 ± 59	9.73 ± 2.95	12.80 ± 1.06	10.20 ± 1.59	2.15 ± 0.48
FDP (day 14)	15.68 ± 1.14	7.01 ± 0.36	314 ± 48	9.35 ± 3.04	11.95 ± 0,93	11.35 ± 2.78	2.55 ± 1.03

b)

Clinical Chemistry	Glucose	Urea	Bilirubin	Creatinine	Protein	Cholesterol
	mmol/L	mmol/L	µmol/L	µmol/L	g/L	mmol/L
Control (pre-dose)	5.30 ± 0.16	5.98 ± 0.59	2.93 ± 0,72	79.00 ± 7.18	56.00 ± 2.34	2.95 ± 0,72
FDP (day 14)	5.28 ± 0.16	5.60 ± 1.10	2.28 ± 0.53	75.75 ± 8.84	65.75 ± 1.48 **	4.53 ± 0.76 *

*$p < 0.05$; **$p < 0.001$

day 14 of the FD phase. In addition, bilirubin was also not elevated compared with predose values. The total protein concentration was significantly higher on day 14 of the FD phase compared to the predose.

Pathology Findings

Organ Weight

Maximum tolerated dose. At termination of this phase, the relative adrenal weights of the beagles were generally higher than normally expected for beagles of this age and strain at this laboratory. Two beagles (one male, one female) had relative brain and lung weights that were considered to be slightly higher than normal, and the relative heart weight of two animals (females) was also considered to be high.

Fixed dose. At the termination of the FD phase of the study, all beagles showed relative higher adrenal weights than normally expected. The relative lung and heart weights of one male and the relative lung weight of one female were considered to be high. The weights of the other organs were considered to be unaffected by treatment.

Gross pathology. Most tissues were unremarkable at necropsy in both MTD and FD phase beagles.

Histopathology. The majority of these findings were of a minor nature and consistent with the expected background pathology in dogs of this strain and age. The only unusual histopathology findings were a mixed, mainly mononuclear, inflammatory perivascular infiltrate cuffing of blood vessels in the medulla oblongata of the brain in all beagles together with a focus of inflammatory cells in the thalamus (one female). The histopathology findings at injection sites (low-grade dermatitis, phlebitis/periphlebitis, and subcutaneous hemorrhage) were consistent with repeated intravenous injections with no evidence of local irritation or other toxicity.

Discussion and Conclusions

The administration of the stabilized orally absorbable form of NADH (ENADA) to beagle dogs at dose levels of 20, 100, and 150 mg/kg/day resulted in very few changes that could be attributed to the compound. There were no deaths. The body weight was unaffected and so was the food consumption. The electrocardiograms did not show any treatment-related changes. The arterial blood pressures, the hematological and the clinical chemistry parameters were not affected by the NADH administration. The composition of the urine was unchanged (data not shown).

The oral administration of NADH to dogs at dose levels of 20, 100, and 150 mg/kg/day elicited apparent increases in adrenal, heart, kidney, liver, brain, and thyroid weights, particularly in the male. This is consistent with the findings from the intravenous administration of NADH, but none of the associated cardiovascular changes, clinical observations, or pathological findings seen in the intravenous MTD and FD toxicity investigations were evident, even after the administration of the highest dose of 150 mg/kg/day stabilized orally absorbable form of NADH for 14 days. The significance of the apparently increased kidney and thyroid weights in the intermediate- and/or high-dose males was unclear, but none of these changes were considered to be of toxicological significance.

The feces of males treated at 150 mg/kg/day became loose or liquid after 3 days of treatment, but this regressed during week 2. There was some evidence of a similar but less marked change in males given a dose of 100 mg/kg/day. However, no corresponding histopathological changes were found in the gastrointestinal tract. Females were unaffected. Therefore, none of these changes were considered to be of toxicological relevance.

It may be argued that the absence of any treatment-related toxic effect could be due to the possibility that NADH is not absorbed in the intestinal tract. Two studies have demonstrated that the stabilized orally absorbable form of NADH (ENADA) is absorbed and passes the intestinal

mucosa undegraded.[22] In addition, oral application of one tablet containing 5 mg NADH (ENADA) lead to an increase of NADH in the brain cortex of rats.[23] This latter study implies that NADH is not only absorbed but also passes the blood–brain barrier. In the meantime, the stabilized orally absorbable form of NADH (ENADA) has been tested in two independent double-blind placebo-controlled trials in people suffering from jet lag and sleep deficiencies.[24,25] Both conditions cause remarkable cognitive impairment, prolonged reaction time, and errors in visual perception. After one dose of oral NADH (20 mg), the improvement in cognitive functions was significantly greater than with placebo but also better than at baseline (before jet lag or sleep deprivation conditions). Another aspect is the rather high concentration of NADH in human organs, particularly those that need a high amount of energy (e.g., heart, brain, and muscle). A mammalian heart contains 90 mg of NADH per kg; the brain and the skeletal muscle, 50 mg per kg.[26] An oral dose of 150 mg NADH per kg amounts to a dose of 10,500 mg for an average weight human of 70 kg. This dose is equivalent to 2100 tablets containing 5 mg NADH (ENADA). This number confirms that NADH (ENADA®) may be generally regarded as safe (GRAS). Furthermore, the recommended daily dosage of ENADA is 1–6 tablets per day, which is many magnitudes below the amount that has been shown to be safe in our study. Studies in humans have revealed that 2 tablets of (ENADA) taken daily over a 4-week period showed therapeutic effects on the symptoms of patients suffering from chronic fatigue syndrome.[3]

To further assess the possible toxicity of NADH, we used the direct route of intravenous infusion. An attempt was made to establish a maximal safe dose in regard to toxicity. The intravenous administration of NADH to beagle dogs at dose levels in the range of 100 and 1000 mg/kg/day resulted in a number of findings that demonstrated an effect on the cardiovascular system. Clinically, these effects included pale gums, pale and cold pads of the feet, bloodshot eyes, and changes in the temperature of the ears. The subdued behavior of the beagles during infusion was also associated with cardiovascular changes. Data from the FD phase showed that blood pressure was lowered 1 hour after the administration of 500 mg/kg/day, and there was an increase in the heart rate that lasted for at least 4 hours after dosing. The heart weights of both MTD phase females and one FD phase male were higher than normally expected. Although this was an indication of an adaptive response to increased workload, there were no histopathological findings in the FD phase experiments to support this.

An increase in adrenal weight was evident in beagles from both MTD and FD phases of the study. Changes in the adrenals could represent a response to stress, but no histopathological changes in the adrenals were found.

There was also evidence of an effect on the central nervous system. At dose levels of 200 mg/kg/day and above the beagles had tremors after dosing, and the relative brain weights of two MTD phase beagles were considered to be higher than normally expected. Microscopic findings in FD phase beagles included an inflammatory cell perivascular cuffing of blood vessels in the medulla oblongata. However, these histopathological findings could not be unequivocally attributed to treatment.

Treatment with NADH at dose levels of 500 mg/kg/day and higher in the MTD phase experiments resulted in a reduction in food intake, with a corresponding decrease in body weight. There were changes in the serum alkaline phosphatase and alanine aminotransferase activities in the serum concentrations of urea, creatinine, cholesterol, calcium, total protein, and total bilirubin compared to the controls, but only the changes in alkaline phosphatase and total cholesterol were significant ($p < 0.05$). The overall significance concerning toxicity of these changes was unclear. Only the activity of serum alkaline phosphatase was above the upper limit of the normal range (190 IU/L).

The FD phase experiments, when 500 mg/kg/day was infused for 14 days, revealed that only the serum cholesterol and protein were significantly elevated compared to predosing levels. However, none of these increases reached pathological concentrations

(normal range: 2.6–6.5 mmol/L; for cholesterol; 5.5–7.5 g/L for total protein).[27] Based on these results, a dose of 500 mg/kg can still be regarded as safe, and the equivalent dose for a 70-kg human individual can be calculated to be 35,000 mg or 35 grams. This amount corresponds to 7,000 NADH (ENADA) 5-mg tablets. Taking into account the remarkably high experimental intravenously administered dosage, NADH can be regarded as a very safe substance.

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