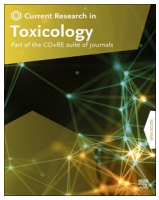
Current Research in Toxicology 6 (2024) 100171

Contents lists available at ScienceDirect

Current Research in Toxicology

journal homepage: www.journals.elsevier.com/current-research-in-toxicology



Toxicology study profile of Nicotinamide mononucleotide after acute and 90-day sub chronic dosing in Wistar rats and mutagenicity tests

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ARTICLE INFO

Keywords:
Toxicology
Chromosomal aberration
Nicotinamide mononucleotide
Uthever ®
Acute oral toxicity

ABSTRACT

Nicotinamide mononucleotide (NMN) is an intermediate in biosynthesis pathway of Nicotinamide adenine dinucleotide (NAD+), an essential cofactor in all living cells involved in fundamental biological processes. Evidence stemming from recent studies have unveiled numerous roles of NAD+ metabolism on aging, longevity, delaying the progression of age-related diseases. A three-study genetic toxicity (genetox) battery (bacterial mutagenesis, in vitro cytogenetics, and in vivo mammalian test) is usually required to confirm safety of a new dietary ingredient and this study showed the data from in vivo mutagenicity test for the first time.

The acute oral LD50 of NMN was greater than 2000 mg/kg body weight with 5000 mg/kg body weight as LD50 cut-off value and was classified under "Category 5 or Unclassified" as per Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Based on 90 days repeated dose toxicity test the NOAEL was considered to be NLT 800 mg NMN/kg body weight in Wistar rats. The bacterial reverse mutation test, the in vitro and in vivo chromosomal aberration test, found NMN to be non-mutagenic. In the mammalian bone marrow chromosomal aberration test, it was concluded that NMN is non clastogenic at and up to 2,000 mg/kg body weight in all the animals tested to confirm safety of a new dietary ingredient and this study showed the data from in vivo mutagenicity test for the first time.

Introduction

Interest of researchers in NMN and its various clinical effects has been on rise ever since it was discovered that NMN is an intermediate in NAD+ biosynthesis pathway in mammals and that NAD+ is the key to aging and related diseases and health conditions (Hsu et al., 2010, Yahyah et al., 2018) (Verdin, 2015; Yoshino et al., 2018; Lautrup et al., 2019). NMN is a nucleotide that is naturally present in human body. With age, the levels of NAD+ and NMN get depleted. Depletion in the levels of NAD+ is associated with traits of aging, many age-related diseases like cancer, metabolic disorders and neurological disorders. Evidence stemming from recent studies have unveiled numerous roles of NAD+ metabolism on aging and longevity and delaying the progression of age-related diseases (Yahyah et al., 2018). Increase in NAD+ levels lead to various health benefits like improvement in cognitive and sensory function, insulin sensitivity in muscle, enhancement of skeletal muscle activity, protective effect in cardio and cerebrovascular diseases. It is also associated with regulation of cellular metabolism including gluconeogenesis in liver, lipogenesis in adipose tissue, insulin secretion in pancreas. NAD+ also promotes endothelial cell proliferation, regulates immune function and protects against acute injury in kidney (Rajman et al., 2018). Enzymes like sirtuins and PARPs i.e. Poly (ADP-ribose) polymerases use NAD+ as co-factor in various signalling pathways. Sirtuins are associated with regulation of inflammation, stress resistance, neuronal functions and cell growth (Gertler and Cohen, 2013; Imai and Yoshino, 2013). PARPS regulate cellular functions like gene expression, DNA repair, cell growth etc.

This has led the researchers to attempt replenishing NAD+ levels with its metabolic substrates. Multiple studies in rodents have shown that systemic NMN administration enhances NAD+ biosynthesis in

Abbreviations: NMN, Nicotinamide Mononucleotide; NAD, Nicotinamide adenine dinucleotide; DRF, Dose Range Finding; DNA, De-oxy Ribonucleic Acid; CPCSEA, Committee for the Purpose of Control and Supervision of Experiments on Animals; OECD, Organisation for Economic Cooperation and Development; GMP, Good Manufacturing Practices; HPLC, High Performance Liquid Chromatography; USP, United States Pharmacopoeia; CAS, Chemical Abstracts Service; CO2, Carbon Di-Oxide; GHS, Globally Harmonized Classification System; NOAEL, no-observed-adverse-effect-level; PCT, Preliminary cytotoxicity test; MMS, Methyl Methane Sulphonate.

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https://doi.org/10.1016/j.crtox.2024.100171
Received 1 October 2023; Received in revised form 22 April 2024; Accepted 2 May 2024
Available online 4 May 2024

various peripheral tissues. (Yoshino et al., 2011; Peek et al., 2013; Stromsdorfer et al., 2016; Karamanlidis et al., 2013; Martin et al., 2017; North et al., 2014; Yamamoto et al., 2014; Gomes et al., 2013; Guan et al., 2017; Lin et al., 2016; de Picciotto et al., 2016). A range of NAD+ boosters are being researched for their safety and efficacy in animals and humans like NMN, NR, nicotinic acid, nicotinamide. NMN has been of particular interest among the lot as it does not produce adverse effects like hepatotoxicity and flushing that nicotinamide does (Poddar et al., 2019). Additionally, NMN was also found to improve insulin resistance, cardiac function, whereas NR doesn't. (Yoshino et al., 2011) NR decreases fatty acid oxidation under exercising conditions, which in turn leads to earlier fatigue. It was also indicated that NR may also disrupt redox homeostasis, which leads cells to a more reductive state. (Kourtzidis et al., 2016)

NMN is also present in some fruits and vegetables like avocado, cabbage, cucumbers etc. in small quantities. For example, 100 g of cabbage contains 0.90 mg of NMN, 100 g of avocado contains 1.6 mg NMN. This is 100 to 300-fold less from what is found to be effective dose in rodent studies. A study in mice found 100 and 300 mg/kg/day dose of NMN to be effective in mitigating the physiological decline with age. In humans, NMN is being explored in doses from 600 mg per day to 1 g per day. (US trial registry no. NCT03151239, NCT04910061, NCT04862338, NCT04823260, NCT04571008, NCT04903210, NCT04664361) Since NMN supplementation is being used to augment the levels of NAD+ in the body, which may go over and above the natural level present, it is imperative to determine the toxic doses and toxic effects of NMN and establish a safety profile.

Various toxicity studies have been carried out on NMN in the past, following is a summary of published non-clinical short term and sub chronic toxicity studies.

Additionally, despite NAD+ contribution in DNA repair, there have been concerns about effect of NAD+ on tumours. This concern stems from the exploration of NAD+ as therapeutic target for cancer therapy. In addition, SIRT1, a metabolic regulator activated by NMN and NR (Gomes et al., 2013) has been shown to have both tumour-suppressive and oncogenic roles, depending on the type and stage of cancer (Chalkiadaki and Guarente, 2015). Since increase in NAD+ may contribute in cell proliferation which may lead to growth of tumour and NMN is associated with replenishing the NAD+ levels in body, there has been a concern about NMN use and cancer. It is worth to evaluate NMN with respect to any carcinogenicity or mutagenic activity due to the differences and complexity of NAD+ contribution in cancer development and as anti-cancer.

We evaluated the safety profile of NMN through acute and 90-day sub chronic dosing in Wistar rats and mammalian cell line. This is the first study to evaluate NMN for mutagenicity, if any, via in vivo mammalian bone marrow chromosomal aberration test along with establishing its safety profile via acute, repeated dose toxicity for 90 days, and in vitro chromosomal aberration tests. These in vitro and in vivo genotoxicity and mutagenicity tests enabled a hazard identification with respect to damage to De-oxy Ribonucleic Acid (DNA) and its fixation.

All studies followed the OECD (Organization for Economic Cooperation and Development) test guidelines and were performed in a Good Laboratory Practice (GLP) – accredited facility at TOXINDIA (Pune, India).

Materials and methods

Test compound

UTHEVER® (CAS No. 1094–61-7) is the registered version of NMN from Effepharm (Shanghai) Co. Ltd. It was manufactured to 99 % purity under GMP conditions by Effepharm (Shanghai) Co. Ltd. The Purity was confirmed on dry weight basis using HPLC. The absence of heavy metals was checked using ICP-MS and microbiology analyses met the U. S. pharmacopeia specifications USP 40. The test item was soluble in distilled water, hence distilled water was used as vehicle in the below mentioned toxicity tests.

Acute oral toxicity test

The study assessed the acute toxic class of NMN with a single dose administered through oral route in 12 healthy female Wistar rats. The study was approved by the Institutional Animal Ethics Committee of TOXINDIA (IAEC Approval Number: TXI/03/01 dated, 31/08/2019). The study was performed according to OECD test guideline No. 423 in 4 steps. Eight to eleven-week-old, nulliparous and non-pregnant female Wistar rats were purchased from Global Bioresearch Solution Private Ltd. Pune, India. The animals were housed in a facility that was maintained at 21 ± 2 ∘C with 45–67 % relative humidity under a 12 −h light/dark cycle where they were fed conventional laboratory pelleted feed from Nutrivet Life Science. Animals were acclimatized to the test conditions for 6 days for step –I, 9 days for step –II, 11 days for step-III and 14 days for step-IV. Test animals were fasted for approximately 17.0 h to 18.0 h prior to dosing. The feed was withheld prior to dosing and approximately 3.0 h to 3.5 h post dosing but drinking water was provided ad libitum. The animals were divided in groups of 3 and received test product through oral gavage of 10 mL/Kg in distilled water. In first step, first group was given the dose of 300 mg/Kg as test dose, since no established dosing information was known for this compound. No mortality was observed, hence, another set of three rats Step II, were dosed with 300 mg/kg body weight. No mortality was observed at this dose level. The next set of three rats Step III were administered with 2000 mg/kg body weight, no mortality was observed. The next set of three rats Step IV were administered with 2000 mg/kg body weight, no mortality was observed. Hence, further dosing was not performed. All animals were observed for mortality and morbidity twice daily. Clinical observations in response to treatment were performed 30 min., 1 h, 2 h, 3 h and 4 h post-administration and once-daily thereafter for 14 days. At the end of 14-day observation period, all the rats were euthanized by overdose of CO_2. All the animals were observed for external and internal gross pathology and change in bodyweight. The study provided information on NMN's LD_{50} and its classification as per the Globally Harmonized Classification System (GHS) (see Table 1 and Table 2).

90-Day repeated dose oral toxicity test

The chronic systemic toxicity of NMN was assessed by administering NMN orally for 90 days to 100 healthy male/female Wistar rats. The study was approved by the Institutional Animal Ethics Committee of TOXINDIA ((IAEC Approval Number: TXI/02/19 dated, 01/12/2018) and followed the Organization for Economic Co-operation and Development (OECD) test guideline 408 "Repeated dose 90-day oral toxicity study in rodents". Eight to Nine-week-old male and female Wistar rats were purchased from Global Bioresearch Solution Pvt. Ltd. Pune, India. The animals were housed in a facility that was maintained at 19 to 23.9 ∘C with 42–61 % relative humidity under a 12 −h light/dark cycle where they were fed conventional laboratory pelleted feed from Nutrivet Life Science. Male rats were acclimatized to the test conditions for 7 days and female rats for 8 days. The animals were randomly allocated to the six groups (10/Sex in main and 5/sex in recovery Group). The doses selected for main groups based on a Dose Range Finding (DRF) study were; 0, 200, 400 and 800 mg/kg body weight and for the recovery groups 0 and 800 mg/kg body weight (Supplement Table 1). Control group animals received vehicle (distilled water) and treatment groups were administered NMN every day by oral gavage, for 90 consecutive days. The recovery groups were not given NMN for 28 days after 90 days of dosing.

Throughout the acclimatization and treatment period, animals were observed for mortality twice per day. Same was followed in recovery period for recovery groups. General clinical observations of rats from the

Table 1
Summary of Published Non-Clinical Short-term and Sub chronic Toxicity Studies with beta-Nicotinamide Mononucleotide.

Species and sex	Test item and Dosing Schedule	Noteworthy findings	Study Limitations	Reference
Rats (Sprague-Dawley) 10/sex in the main study and 5/sex in the recovery groups	NMN-C® compound (99.03 % purity) 0,375,750 or 1500 mg/kg bw/day gavage administered for 90 days with a 28-day recovery period	No significant treatment-related adverse effects on measured parameters NOAEL =>1500 mg/kg bw/day	• The test item is not representative of the novel food that is subject of this application. • Doses were markedly higher than those used in the 90-day study commissioned by Effepharm which were selected specifically to support the intended use level of beta-NMN in food supplements.	Cros *et al.* (2021)
Male mice (C57BL6J); group size not reported.	NMN (no other details were provided). 0,1,340,or 2,680 mg/kg bw/day for 7 days	Generally well-tolerated. Compared to the control group, low-dose mice had significantly decreased body weight and significantly decreased ratio between liver and body weight. A significant decrease in the ratio between liver and body weight was also observed in high-dose mice. High dose mice had a significant increase in alanine aminotransferase, but no liver histopathological effects were noted.	• The test item is not representative of the novel food that is subject of this application. • No female animals included. • Animal group size was not reported. • Not conducted in compliance with OECD Test Guidelines or GLP.	You et.al. (2020)
Dog (Beagle); 5/group	NMN (no other details were provided) 0 or 1,340 m/day for 2 weeks	Generally well-tolerated. Mild increase in creatinine and uric acid, indicating a kidney response.	• The test item is not representative of the novel food that is subject of this application. • Sex not specified. • Not conducted in compliance with OECD Test Guidelines or GLP. • Conducted in dogs.	You et.al. (2020)

bw = body weight, GLP = Good Laboratory Practice; NMN = nicotinamide mononucleotide; NOAEL = no observed-adverse-effect level; OECD = Organisation for Economic Co-operation and Development.

Table 2
Following is a summary listing of some of In vitro Genotoxicity Studies of *beta*-Nicotinamide Mononucleotide.

Test	Test System/ Animal Species	Concentration/ Dose	Results	Reference
Bacterial reverse mutation test	*Salmonella typhimurium* TA98, TA100, TA1535, TA1537; *E.coli* WP2 (pKM101)	Up to 5,000 µg/plate +/- S9	Negative	Cros *et al.* (2021)
	Salmonella typhimurium TA98, TA100, TA1535, TA1537; *E.coli* WP2 (uvrA)	Up to 5,000 µg/plate +/- S9	Negative	Fukamizu *et al.* (2022)
Micronucleus test	Chinese hamster ovary cells	Up to 2,000 µg/mL +/- S9	Negative	Cros *et al.* (2021)

+ S9 = with metabolic activation; − S9 = without metabolic activation

all groups were made once a day. Body weight, feed consumption, detailed clinical examination was done prior to the start of treatment and weekly, throughout the treatment and recovery period. At week 13th and week 17th (only for recovery groups), ophthalmological, functional, clinical pathology (biochemistry and haematology) examinations were done. All the surviving animals were euthanized using excess CO2 at the end of dosing period and necropsy and gross macroscopic examinations were done.

Bacterial reverse mutation test

The bacterial reverse mutation test assessed the mutagenic potential of NMN in several bacterial strains. The test was performed in accordance with OECD Test Guideline 471. Bacterial strains (*Salmonella typhimurium*) used were TA98, TA100, TA1535, TA97a and TA102, obtained from Moltox, USA.

A Preliminary Cytotoxicity Test (PCT) was performed as plate incorporation method with eight test concentrations viz. 5000, 2500, 1250, 625, 312,5, 156.25, 78.125 and 39.0625 ug/plate in presence (5 % S9) and absence of metabolic activation system using tester strains of TA98 and TA100 in triplicate.

The Main study was performed as Trial I by plate incorporation method using file tester strains with (5 % S9) and without metabolic activation and Trial II by pre-incubation method using all five tester strains with (10 % S9). All the plates Trial I and Trial II study were maintained in triplicates for each concentration.

Since, the test item was found to be non-cytotoxic in Preliminary Cytotoxicity Test, the doses for main study were selected with approximately half log difference ($\sqrt{10}$) interval. The doses selected for main study were 5000, 1500, 500, 150, and 50 ug/plate in presence and absence of metabolic activation (S9) system. The strains were exposed to test concentrations, vehicle or positive controls and poured onto minimal glucose agar medium supplemented with L-histidine and D-biotin. The plates were incubated at approximately for 37 °C for about 48 h, after which the revertant colonies were counted and their frequency was compared with that in the concurrent vehicle control.

In vitro mammalian chromosomal aberration test

This in vitro test assessed the potential of NMN to cause structural chromosomal aberrations in cultured mammalian cells in accordance with OECD test guideline 473. The Chinese Hamster Ovary (CHO) cells (procured from National Centre for Cell Sciences, Pune, India) were exposed to positive controls or NMN concentrations in presence and absence of a metabolic activation system (S9 prepared in house). Selection of NMN test concentrations for the chromosome aberration assay was based on cell growth inhibition relative to the solvent control in

Preliminary Cytotoxicity Test (PCT). Prior to treatment, CHO (Chinese Hamster Ovary) cells were seeded with MEM, 10 % Fetal bovine serum, penicillin streptomycin in a tissue culture flask to achieve 2.5 x 105 cells/ml. To determine the clastogenic activity of NMN, cells were exposed to 500, 1000, 2000 ug/ml of NMN, water or the appropriate positive control (Cyclophosphamide monohydrate and Methyl methane sulphonate), the cultures were incubated at 37 ± 1 °C temperature and 5 ± 1 % CO2 in a humidified atmosphere in a CO2 incubator for 3 h in the metabolic activation system and in the non-activated test system for 3 and 20 h.

After treatment, cells were treated with Colchicine (5 μg/mL) for 3 h to block mitosis at metaphase, and then Giemsa solution (5 %) was used for staining. Chromosome aberrations were observed in well-spread metaphases 300 per concentration of test substance.

***Mammalian bone marrow chromosomal aberration** (in-vivo)*

This in vivo test assessed the potential of NMN to cause structural chromosomal aberrations in 35 healthy female Swiss albino mice rats [obtained from Global Bioresearch Solution Pvt. Ltd. Pune, INDIA (CPCSEA Register No.:1899/PO/Bt/S/16/CPCSEA)] in accordance with OECD test guideline 475. The study was approved by the Institutional Animal Ethics Committee of TOXINDIA (IAEC Approval Number: TXI/02/21 dated, 01/12/2018). The rats were nulliparous, non-pregnant and 6 weeks old at time of dosing. The animal housing facility was maintained at 20.9 to 23 ∘C with 45–62 % humidity under a 12 −h light/dark cycle. Conventional laboratory pelleted feed from Nutrivet Life Science and water were supplied ad libitum. Methyl Methane Sulphonate (40 mg/Kg body weight, obtained from Sigma Aldrich, India) was used as positive control in the study. The acclimatized animals were given NMN/MMS through oral gavage (10 mL/Kg body weight) in doses of 500, 1000 and 2000 mg/kg body weight, based on a DRF study using both male and female mice (Supplement Table 2). The main study was done only using female mice as there was no difference in systemic toxicity between both sexes. Dosing of control/test item was done on day one followed by intra- peritoneal colchicine administration at 21 h after dosing, and samples were collected at 24 h after sacrificing animals. Bone marrow cells from both the femora were taken out, flushed with hypotonic saline (20 min), pelleted and re-suspended in Carnoy's fluid. Once again the cells were pelleted and dropped on clean glass slides with a Pasteur pipette. Giemsa staining was done and increased number of aberrations in metaphase chromosomes (minimum 100) was used as the evaluation criteria. All animals were also observed for mortality and morbidity twice daily throughout acclimatization and post dosing. Clinical observations in response to treatment were performed 30 min, 1 h, 2 h, 3 h and 4 h post-administration, on the day of dosing and prior to cervical dislocation. Body weight of all the animals was taken before acclimatization, during group randomization, before dosing and before sacrifice.

Statistical analysis

Statistical Analysis for 90-day repeated dose study was performed using Graph Pad Prism. Body Weight, Body weight change, Feed Consumption, haematology, clinical biochemistry, T3, T4, TSH, and Organ Weight etc. Data was subjected to parametric one-way analysis of variance (ANOVA). If ANOVA revealed statistical significance ($P < 0.05$) then Dunnett's Test was used to compare test item treated group to control. Standard deviation and Number of observation was used to calculate mean.

In the in vitro mammalian chromosomal aberration test, the percentage of cells with structural aberrations was considered as basic unit for statistical analysis. For main study statistical analysis was performed for structural aberrations with respect to percent cell growth inhibition and occurrence of acentric fragments, deletions dicentrics, breaks and gaps in chromosome and chromatid in treated cells to the results obtained for the vehicle control group using "Student's t-test" at 95 % confidence interval.

For in vivo mammalian bone marrow chromosomal aberration test, approximately 200 metaphases were analysed for each animal for structural chromosomal aberrations including and excluding gaps. Chromatid and chromosome type aberrations were recorded separately and classified by subtypes (breaks exchanges fragments and deletions). To describe toxicity, effect the mitotic index (percent of cells in mitosis) was determined in 2000 cells scored. The results were obtained using Chi-square test.

Results and discussion

Nicotinamide adenine dinucleotide (NAD) one of the most important and interesting molecules in the body, required for over 500 enzymatic reactions and plays key roles in the regulation of almost all major biological processes (Ansari and Raghava, 2010).

Emerging studies have heightened the fact that the cellular level of NAD+ decreases with age, predisposing individuals to physiological decline as well as late-onset diseases (Rajman et al., 2018). Thus, enhancing NAD+ availability by supplementing precursory metabolites, such as NMN and NR, promises to ameliorate a broad spectrum of age-associated deficits (Partridge et al., 2020).

Various efforts have been made to explore the therapeutic benefits of NMN in rodents, the clinical and toxicological evidence to support NMN safety at high doses still remains insufficient.

Acute oral toxicity test

No mortality was observed in any of the animals throughout the 14 days' observation period. At 300 mg/kg body weight (Step I and II) and at 2000 mg/kg body weight (Step III and IV), all animals were observed normal with respect to clinical observations throughout study period. The mean body weight of all the animals were observed with gain on day 7 and 14, as compared to day 0 (Supplement Table 3) (Fig. 1). No external and internal gross pathological changes were seen in any of the animals of Step I and II treated with 300 mg/kg body weight and Step III and Step IV treated with 2000 mg/kg body weight.

The body weight of all rats was observed throughout for a period of 14 days. The mean body weight at day 7 and day 14 was compared with day 0.

Acute oral toxicity study on NMN showed no mortality even at a dose level of 2000 mg/kg body weight. Body weight is one of the most sensitive indicators of the condition of the animal. A treatment dose which causes more than 10 % reduction in body weight is considered to be toxic *(Kushwaha et al., 2013)*. Our results confirmed that the study product did not possess any acute oral toxicity in mice.

90-Day repeated dose oral toxicity test

In the past 90-day sub-chronic toxicity studies on NMN at doses of

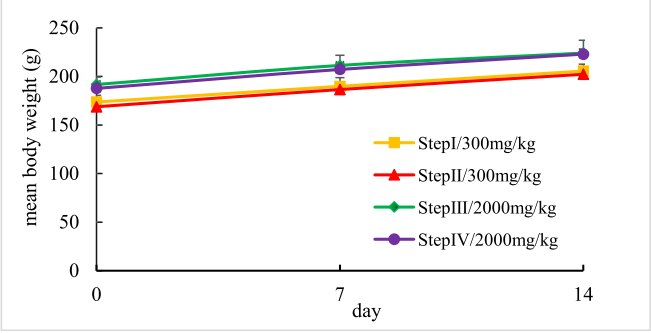

Fig. 1. Effects of single dose of NMN on body weight in rats.

375, 750 and 1500 mg/kg/d followed by a 28-day treatment-free recovery period, have appeared to be safe with no toxic effects as indicated by body weight change, food and water consumption, feed conversion efficiency, biochemical and blood parameters as well as organ toxicity and histological examinations of main organs.

During the 90-day sub chronic toxicity study on NMN no statistically significant difference in the body weight and body weight change was observed in the treatment as compared to control group during study period. Statistically significant difference was observed in some of the physiological and biochemical parameters, but were of minor magnitude and not considered as treatment related due to lack of dose dependency and inconsistency between sexes. Individual observations have been summarized.

Survival, clinical observations and ophthalmologic examination

No mortality was observed in any of the dose groups, during the entire study period including recovery period. Ophthalmological examination did not reveal any test item related changes in both the eyes at the end of treatment periods, in any of the animals observed, when compared with concurrent control group animals (data not shown).

Body weight and body weight gain

There was no statistically significant difference in the body weight and body weight change of G2, G3, G4 and G4R as compared to control group G1 and G5 respectively during study period whereas statistical significant decrease in percent body weight change of G3 and G4 female on day 1–85 and of G3 female on day 1–90 as compared to G1 (Fig. 2). This change is inconsistent and not related to test item.

The mean body weight of all rats were observed throughout for a period of 90 days.

Food consumption and efficiency

There was no statistically significant difference in average feed consumption (gram/day/ animal) in all treated group (G2, G3, G4, G6) as compared to control group (G1 and G5) respectively during the study period (Supplement Table 4–6).

Sensory activity

The sensory reactivity measurements recorded during this study are commonly observed in the test system and were not attributed to the test item. Foot splay, Motor activity and fore limb and hind limb grip strength parameters were comparable and no treatment related changes were revealed in any of the animals of all treated groups whereas statistical significant increase observed in G6 female motor activity as compared to G5. Statistical significant increase in G2 female foot splay as compared to G1 and in G6 female forelimb grip strength as compared to G5 (Supplement Table 7). This change is inconsistent, not dose dependent and hence not related to test item.

Clinical pathology: hematology, coagulation, clinical chemistry, and urinalysis

All Haematology parameters estimated were unaltered by test item treatment (Table 3). Statistical significant increase in Neutrophil, MCV and Monocyte of G3, decrease in Lymphocyte of G3 male as compared to

Table 3
Summary of Clinical Haematology Data.

Sex	Male		Female	
Group (N) Parameters	G1 (10) Mean ± SD	G5 (5) Mean ± SD	G1 (10) Mean ± SD	G5 (5) Mean ± SD
WBC	11.99 ± 1.82	9.17 ± 4.68	10.45 ± 2.57	5.89 ± 2.33
Neu%	17.55 ± 6.31	23.2 ± 3.75	18.2 ± 2.32	24.6 ± 5.68
Lym%	74.8 ± 9.28	63.4 ± 4.07	74.1 ± 3.73	65.9 ± 7.07
Mon%	5.0 ± 3.30	4.56 ± 1.88	4.9 ± 2.38	5.12 ± 3.01
Eos%	0.06 ± 0.10	0.21 ± 0.09	0.09 ± 0.13	0.10 ± 0.12
Bas%	0.16 ± 0.05	0.16 ± 0.09	0.15 ± 0.06	0.26 ± 0.11
RBC	8.18 ± 0.39	7.34 ± 0.41	7.39 ± 0.31	6.87 ± 0.51
HGB	16.1 ± 0.83	16.1 ± 0.96	15.2 ± 0.61	15.4 ± 0.99
HCT	38.11 ± 2.26	38.8 ± 2.10	41.1 ± 1.70	37.9 ± 2.58
MCV	47.23 ± 0.84	49.2 ± 0.61	51.2 ± 1.11	50.1 ± 0.69
MCH	17.22 ± 0.31	18.92 ± 0.39	18.7 ± 0.42	19.5 ± 0.61
MCHC	36.2 ± 0.46	36.9 ± 0.48	35.7 ± 0.41	37.2 ± 0.80
ROW-CV	17.9 ± 2.02	18.1 ± 1.63	15.2 ± 2.47	16.30 ± 2.44
PLT	522.8 ± 187.03	587.2 ± 92.34	679.2 ± 41.42	799.1 ± 58.93
No. Reticulocyte	18 ± 2.35	23.2 ± 3.56	19.3 ± 2.49	22.3 ± 2.41
Reticulocyte (%)	1.8 ± 0.23	2.32 ± 0.35	1.93 ± 0.25	2.23 ± 0.24
Clotting time (Sec.)	130.2 ± 7.41	123.2 ± 3.85	113.2 ± 5.80	118.3 ± 3.63

The following statistically significant differences (in comparison to the control group) in haematological parameters were considered unrelated to the test item by the study authors, as they were not associated with a dose response and there were discrepancies between sexes: increased neutrophils, monocytes, and mean corpuscular volume in mid-dose males; decreased lymphocytes in mid-dose males; decreased mean corpuscular haemoglobin concentration in mid- and high-dose males; increased neutrophils and haematocrit in mid- and high-dose females; decreased lymphocytes and platelets in mid- and high-dose females; and increased red blood cells, haemoglobin, and mean corpuscular haemoglobin concentration in high-dose females. In recovery animals, a statistical decrease in eosinophils was noted in males compared to the control group. However, there were no differences in the above significant effects at the end of the recovery period.

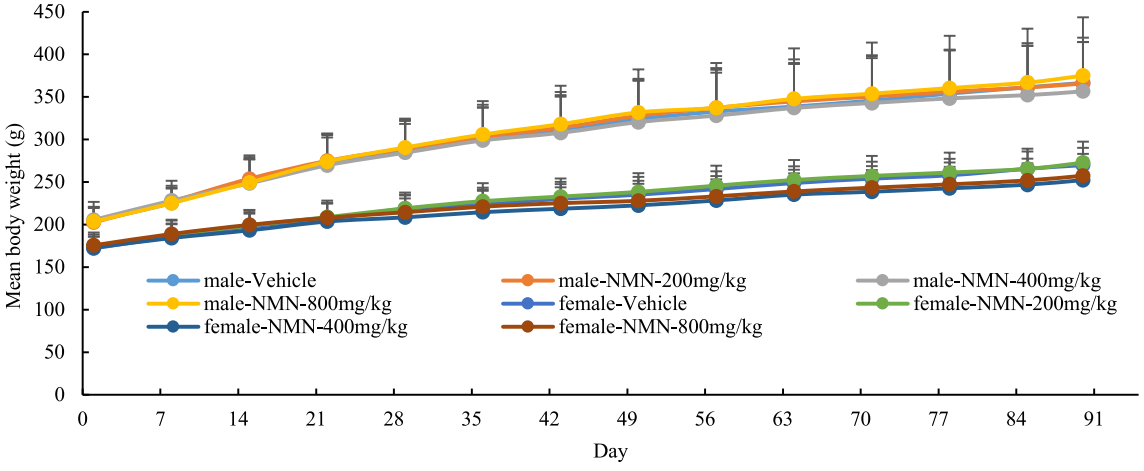

Fig. 2. Body weight changes of rats in the 90-day oral toxicity study of NMN.

G1. Statistical significant decrease in MCHC of G3 and G4 male as compared to G1. Statistical significant increase in Neutrophil of G3 and G4 female, decrease in Lymphocyte of G3 and G4 female as compared to G1. Statistical significant increase in RBC of G4 female, HGB of G4 female, HCT of G3 and G4 female, MCHC of G4 female, decrease in platelet of G3 and G4 as compared to G1. Statistical decrease in eosinophil of G6 male as compared to G5. Review of the data at the end of treatment period did not reveal statistically significant variations in any of the observed biochemistry parameters (Table 4), except increased albumin and cholinesterase of G2 male as compared to G1. Statistical decrease in Total bilirubin, Total protein, chloride of G3 male as compared to G1. Statistical decrease in Creatinine, LDH (lactose dehydrogenase), Aspartate aminotransferase, chloride of G4, increase in sodium of G4 male as compared to G1. Statistical decrease in cholesterol of G4 female, cholinesterase of G2 female, increase in G3 female LDH and Aspartate aminotransferase as compared to G1. Statistical decrease in chloride of G3 and G4 female, increase in sodium of G3 and G4 female as compared to G1. Statistical decrease in triglyceride of G6 female, increase in sodium of G6 female as compared to G5. Statistical significant decrease in chloride of G6 male, increase in blood urea nitrogen of G6 male as compared to G5. These changes were not considered as treatment related effect due to lack of dose dependency and inconsistency between sexes.

Necropsy and pathology

External and internal gross examination of all the animals did not reveal any abnormality of pathological significance. Absolute and relative organ weights of all test item treated animals were unaffected when compared to control group. Statistical significant increase in absolute weight of G4 male Brain, Epididymides, Seminal vesicle and prostate as compared to G1, decrease in G4 female thyroid as compared to G1, decrease in G6 female kidney as compared to G5. Statistical significant increase in relative weight of G4 male epididymides, seminal vesicle and prostate as compared to G1 (Table 5). Statistical significant increase in relative weight of G4 female thyroid and adrenal and decrease in brain as compared to G1. Statistical significant decrease in relative weight of G6 male heart and G6 female kidney as compared to G5. Microscopic lesions observed in liver, lung, kidneys of control and high dose treated groups are minimal to in nature. Further these observed lesions are common in occurrence in rodents during toxicological studies (Boorman et al., 1990, Greenman et al., 2007; Greaves and Faccini, 1992; Haschek et al., 2002). Hence, occurrence of these lesions could be considered as spontaneous or incidental in nature.

Based on the findings of study 90 days repeated dose oral toxicity study of NMN in rat with 28 days' recovery, where in 0, 200, 400 and 800 mg/kg body weight, doses were tested; No test item related changes were observed in G4 (800 mg/kg body weight), hence the no Observed Adverse Effect Level (NOAEL) of NMN is considered to be 800 mg/kg body weight in rat under the experimental conditions.

Bacterial reverse mutation test

Results of present study indicated that NMN was not cytotoxic at any of the doses used in this study, and compared to the vehicle control, did not increase the number of revertant colonies in any of the tester strains either when incubated in the presence or absence of the S9 mix, or using the plate incorporation or pre-incubation methods (Supplement Table 8). In contrast, all positive controls (2-Aminofluorene, sodium azide, 4-nitroquinoline-N-oxide, methyl methane sulphonate) significantly increased in the number of revertant colonies ($p < 0.05$), demonstrating both the sensitivity and validity of the assay. Therefore, NMN was not mutagenic under the conditions used in the studies. The results of each colony (using plate incorporation and pre-incubation respectively) are summarized in Supplement Table 8 and Table 6.

Table 4
Summary of Clinical Chemistry Data.

Sex	Male		Female	
Group (N) Parameters	G1 (10) Mean ± SD	G5 (5) Mean ± SD	G1 (10) Mean ± SD	G5 (5) Mean ± SD
Albumin (g/dl)	3.65 ± 0.12	3.70 ± 0.09	3.66 ± 0.17	3.57 ± 0.12
Alkaline Phosphatase (U/1)	460.8 ± 144.63	502.16 ± 180.81	3208 ± 163.08	245.56 ± 52.84
Bilirubin Direct	0.11 ± 0.02	0.08 ± 0.01	0.12 ± 0.02	0.09 ± 0.04
Bilirubin Total (mg/dl)	0.10 ± 0.02	0.11 ± 0.01	0.15 ± 0.02	0.12 ± 0.03
Calcium (mg/dl)	10.22 ± 0.23	10.82 ± 0.23	9.94 ± 0.27	9.99 ± 0.27
Cholesterol (mg/dl)	61.5 ± 10.71	51.3 ± 8.82	55.8 ± 3.16	49.44 ± 10.46
Cholinesterase (U/l)	1246 ± 139.35	1099.3 ± 62.68	2206.8 ± 562.71	1790.04 ± 366.48
Creatinine (mg/dl)	0.49 ± 0.05	0.58 ± 0.06	0.53 ± 0.04	0.77 ± 0.08
Glucose (mg/di)	113.4 ± 18.85	112.4 ± 18.21	88.9 ± 17.85	97.4 ± 21.55
Lactate Dehydrogenase (U/l)	1370.23 ± 314.10	1849.0 ± 364.84	930.44 ± 297.60	1643.92 ± 281.39
Phosphorus (mg/dl)	4.98 ± 0.73	5.42 ± 0.44	4.11 ± 0.66	4.13 ± 1.14
Aspartate aminotransferase (U/l)	187.5 ± 30.88	176.9 ± 30.05	126.0 ± 26.54	159.2 ± 18.28
Alanine Aminotransferase (U/l)	56.7 ± 9.74	67.0 ± 3.99	58.9 ± 15.23	48.3 ± 8.30
Total Protein (mg/dl)	7.21 ± 0.33	7.76 ± 0.24	7.53 ± 0.35	6.98 ± 0.34
Triglycerides (mg/dl)	96.2 ± 35.78	124.5 ± 24.85	77.8 ± 23.33	80.3 ± 6.88
Urea (mg/dl)	40.6 ± 4.52	40.0 ± 1.63	41.3 ± 7.16	44.7 ± 0.53
Calculated Blood Urea Nitrogen (mg/dl)	16.9 ± 2.11	19.20 ± 0.76	15.80 ± 3.34	20.88 ± 0.25
Sodium (mmol/l)	141.4 ± 1.96	138.99 ± 0.44	143.59 ± 1.72	150.5 ± 1.32
Potassium (mmol/l)	4.01 ± 0.23	3.65 ± 0.32	4.11 ± 0.21	4.96 ± 0.48
Chloride (mmol/l)	97.3 ± 2.12	101.9 ± 1.33	98.0 ± 1.63	98.60 ± 1.32
Bile Acid	0.06 ± 0.02	0.06 ± 0.01	0.08 ± 0.02	0.07 ± 0.02

The following statistically significant differences (in comparison to the control group) in blood chemistry parameters were considered unrelated to the test item by the study authors, as they were not associated with a dose response, fell within historical control data, and there were discrepancies between sexes: increased albumin and cholinesterase in low-dose males; decreased total bilirubin, protein, and chloride in mid-dose males; decreased lactate dehydrogenase, creatinine, chloride, and aspartate aminotransferase in high-dose males; increased sodium in high-dose males; decreased cholesterol in high-dose males; decreased cholinesterase in low-dose females; increased lactate dehydrogenase aspartate aminotransferase and decreased chloride in mid- and high-dose females; and increased sodium in mid- and high-dose females. In recovery animals, a statistical decrease in triglyceride and increase in sodium were noted in female rats, and a statistical decrease in chloride and increase in blood urea nitrogen were noted in male rats. When comparing the high-dose animals and the recovery animals, the decrease in chloride in males and the increase in sodium in females were consistent between these groups, but still fell within historical control data from the laboratory. Thyroid hormones were unaffected by the test item.

In vitro mammalian chromosomal aberration test

NMN was not cytotoxic to ex vivo CHO cells at any of the concentrations used in the study as determined by the % cell growth inhibition (data not shown), and, compared to the vehicle control, NMN did not increase the number of aberrant metaphases when incubated with S9 for 3 h and without S9 for 3 (Supplement Table 9) and 20 h (Table 7).

Table 5
Summary of Absolute organ weight.

Sex	Male					
Group (N)	**G1 (10)**	**G2 (10)**	**G3 (10)**	**G4 (5)**	**G5 (5)**	**G6 (5)**
Parameters	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Body weight	352.38 ± 53.38	351.95 ± 47.62	343.91 ± 59.57	362.32 ± 68.40	389.00 ± 36.19	385.78 ± 73.77
Heart	1.1071 ± 0.1526	1.0689 ± 0.1096	1.0851 ± 0.1879	1.0865 ± 0.2123	1.2323 ± 0.1890	0.9963 ± 0.1984
Spleen	0.6766 ± 0.1200	0.7021 ± 0.1329	0.6991 ± 0.1977	0.7520 ± 0.1906	0.7295 ± 0.0714	0.6426 ± 0.1804
Liver	11.5381 ± 1.7415	10.9050 ± 2.3503	11.3583 ± 3.5331	12.3728 ± 3.4588	10.8006 ± 1.2486	10.5954 ± 1.7010
Kidneys	2.6014 ± 0.3599	2.5631 ± 0.4494	2.6684 ± 0.5316	2.8745 ± 0.6292	2.4610 ± 0.2129	2.4552 ± 0.4605
Brain	2.0223 ± 0.1476	2.1310 ± 0.1106	2.1907 ± 0.2109	2.2391 ± 0.1475	2.2482 ± 0.1856	2.1500 ± 0.1926
Testes	3.1214 ± 0.3204	2.9851 ± 0.1908	3.0038 ± 0.3999	3.1086 ± 0.3139	3.0822 ± 0.1304	3.9541 ± 2.4083
Ptuitary	0.01845 ± 0.00687	0.02318 ± 0.00920	0.02321 ± 0.00814	0.02329 ± 0.01109	0.01750 ± 0.00404	0.01846 ± 0.00589
Thyroid/Parathyroid	0.02501 ± 0.00277	0.02380 ± 0.00297	0.02924 ± 0.00771	0.02851 ± 0.00700	0.01770 ± 0.00295	0.01752 ± 0.00331
Thymus	0.1728 ± 0.0442	0.1696 ± 0.0436	0.1517 ± 0.0333	0.1963 ± 0.0637	0.1396 ± 0.0244	0.3173 ± 0.4931
Adrenals	0.04521 ± 0.00708	0.04950 ± 0.01050	0.05051 ± 0.00900	0.05453 ± 0.01502	0.04006 ± 0.00296	0.04557 ± 0.01094
Epididymides	1.2912 ± 0.1538	1.2527 ± 0.1513	1.2836 ± 0.2319	1.5417 ± 0.2056	1.1464 ± 0.1990	1.2116 ± 0.1283
Seminal vesicle and prostate	1.5665 ± 0.4908	1.8831 ± 0.3519	1.6975 ± 0.3791	2.1976 ± 0.5317	1.6749 ± 0.7797	1.8503 ± 0.6646
Sex	**Female**					
Group (N)	**G1 (10)**	**G2 (10)**	**G3 (10)**	**G4 (5)**	**G5 (5)**	**G6 (5)**
Parameters	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Body weight	257.91 ± 20.60	260.20 ± 24.23	239.44 ± 24.48	245.36 ± 25.42	282.64 ± 11.53	273.93 ± 18.72
Heart	0.8208 ± 0.0841	0.8215 ± 0.1163	0.7442 ± 0.1225	0.7684 ± 0.0901	0.8099 ± 0.1087	0.7377 ± 0.1500
Spleen	0.4839 ± 0.0862	0.5184 ± 0.1131	0.4645 ± 0.0793	0.4403 ± 0.1033	0.3988 ± 0.0651	0.3689 ± 0.0756
Liver	7.0776 ± 0.8581	7.1442 ± 1.4018	6.4986 ± 0.7412	7.1646 ± 1.2713	6.7960 ± 1.2162	5.4651 ± 0.9593
Kidneys	1.7121 ± 0.2297	1.8258 ± 0.2592	1.7127 ± 0.2228	1.7854 ± 0.3182	1.6823 ± 0.1452	1.3940 ± 0.2083
Brain	2.0624 ± 0.0546	2.0590 ± 0.1622	2.1548 ± 0.1013	1.9641 ± 0.1324	2.0522 ± 0.1482	1.9548 ± 0.1159
Ovaries	0.09717 ± 0.01491	0.18347 ± 0.32330	0.08244 ± 0.01913	0.07157 ± 0.01173	0.06855 ± 0.00731	0.07266 ± 0.00713
Ptuitary	0.03027 ± 0.04060	0.02314 ± 0.00656	0.02361 ± 0.00485	0.02029 ± 0.00814	0.02061 ± 0.00485	0.1513 ± 0.00482
Thyroid/Parathyroid	0.03068 ± 0.00537	0.02814 ± 0.00908	0.02012 ± 0.00331	0.02380 ± 0.00297	0.01571 ± 0.00298	0.01689 ± 0.00307
Thymus	0.1417 ± 0.0492	0.1651 ± 0.0473	0.1443 ± 0.0517	0.1781 ± 0.0614	0.1105 ± 0.0334	0.1280 ± 0.0367
Adrenals	0.06391 ± 0.01608	0.06048 ± 0.01066	0.05495 ± 0.01053	0.05112 ± 0.01529	0.06246 ± 0.00640	0.05361 ± 0.01062
Uterus	0.5176 ± 0.1128	0.5249 ± 0.0848	0.4918 ± 0.0996	0.5108 ± 0.2031	0.5409 ± 0.0668	0.5185 ± 0.1148

The following statistically significant differences (in comparison to the control group) were reported for absolute organ weight: increased weight of the epididymides, seminal vesicles, and prostate in high-dose males; decreased thyroid weight in high-dose females; and decreased weight of the kidneys in female recovery animals. However, there were no test item-related macroscopic, microscopic, or histopathological findings observed.

Table 6
Ames Assay Results (Pre-incubation method).

	Test Item	Test concentrations (ug/plate)	Mean Colonies/Plate (mean ± SD)				
			TA1535	TA97a	TA98	TA100	TA102
Pre-incubation Method							
With S9 Mix	Water	–	12.67 ± 3.06	128 ± 7.55	42.00 ± 4.00	168.00 ± 6.00	282.00 ± 4.36
	NMN	5000	8.00 ± 2.00	118.33 ± 2.52	34.00 ± 2.00	156.67 ± 4.16	271.33 ± 2.52
		1500	5.33 ± 1.15	121.33 ± 10.07	34.33 ± 3.51	160.00 ± 3.00	270.67 ± 2.08
		500	9.33 ± 2.52	116.00 ± 6.00	35.33 ± 2.52	152.33 ± 6.11	261.00 ± 2.65
		150	9.33 ± 4.04	127.33 ± 3.06	36.00 ± 3.61	154.00 ± 8.54	264.33 ± 4.73
		50	9.33 ± 1.15	118.67 ± 5.03	36.33 ± 1.15	157.33 ± 3.79	278.00 ± 2.65
	2-Aminofluorene	10	193.67 ± 14.57	356.00 ± 18.73	173.67 ± 5.69	501.67 ± 11.15	1341.67 ± 63.32
Without S9 Mix	Water	–	11.33 ± 2.52	128.67 ± 3.06	44.00 ± 3.00	176.00 ± 4.58	290.67 ± 2.52
	NMN	5000	6.00 ± 2.65	122.00 ± 4.36	34.67 ± 2.08	152.33 ± 3.51	277.67 ± 2.52
		1500	8.00 ± 3.00	120.00 ± 5.29	34.00 ± 2.65	156.00 ± 5.57	277.33 ± 3.51
		500	6.00 ± 2.00	123.33 ± 4.04	36.67 ± 3.06	153.67 ± 4.93	265.00 ± 7.00
		150	8.33 ± 3.06	124.67 ± 2.08	34.67 ± 1.15	152.67 ± 5.03	266.67 ± 6.51
		50	11.00 ± 2.65	123.00 ± 3.61	35.33 ± 3.06	153.33 ± 3.51	278.00 ± 2.65
	Sodium Azide	1.5	183.00 ± 10.15	–	–	489.33 ± 12.06	–
	4-Nitroquinolene-N-Oxide	0.5	–	334.33 ± 16.50	153.00 ± 4.58	–	–
	Methyl Methane Sulphonate	1	–	–	–	–	1274.67 ± 67.90

Table 6 shows the results for Bacterial Reverse Mutation test using Ames Assay Pre-incubation method. The test used various concentrations of NMN and positive and negative controls. The data (Mean Colonies/Plate) are shown as mean ± SD.

Moreover, the types of aberrations detected in the vehicle- and NMN-treated cells were similar. In contrast, the positive controls, methyl methane sulphonate and cyclophosphamide monohydrate significantly increased the number of aberrant metaphases ($p < 0.05$), thus confirming the sensitivity and validity of the assay. Therefore, under the conditions of this study, NMN was not clastogenic at and up to 2000 ug/ml in CHO cells.

Mammalian bone marrow chromosomal aberration

The study tested NMN at concentrations of 2000, 1000 and 500 mg/kg body weight. The animals were not found in a moribund condition nor showing in severe pain or signs of enduring severe distress. Mortality was not found and clinical observations were found to be normal. The weight variation of animals was minimal and did not exceed ± 20 % of mean body weight.

Table 7
Summary of Results-In Vitro Chromosomal Aberration Assay in Chinese Hamster Ovary Cell line-20-hour Treatment.

	Dose Level of Test Medium (ug/ml)					
	Untreated	Vehicle control[a]	NMN 500	NMN 1000	NMN 2000	Positive Control[b]
Without S9						
Total metaphases scored	400	300	300	300	300	100
Total aberrations	3	2	3	5	2	11
Including gaps	3	2	3	5	2	11
Excluding gaps	3	2	3	5	2	11
The percentage of cells with structural aberrations	0.75	0.67	1.00	1.67	0.67	11.00

Table 7 shows results for In vitro Mammalian Chromosomal Aberration Test in Chinese Hamster Ovary Cell line-20 h Treatment without metabolic activation for the following: untreated, vehicle control, Positive control and 3 concentrations of NMN. The table presents total metaphases, total aberrations, percentage of cells with structural aberrations.

[a] : Vehicle control = distilled water.

[b] : Positive control = Methyl methane sulphonate (30ug/ml) in the absence of metabolic activation (without S9).

The percent mitotic index in vehicle control group was 8.08 % while the test item showed 6.94, 7.06 and 7.24 % at the dose levels of 2000, 1000 and 500 mg per kg body weight respectively. Concurrent positive control methyl Methane sulphonate group showed 5.42 % mitotic index at the dose level of 40 mg per Kg body weight.

The mean chromosomal aberration frequency per group at all tested concentrations of test item was found to be comparable to those observed in the vehicle control group. The mean chromosome aberration frequency in vehicle control group was 1.20 while the test item showed 1.40 1.00 and 1.40 at the dose levels of 2000, 1000 and 500 mg per kg body weight respectively. The reversal group of highest dose (2000 mg/kg body weight) showed 1.00 frequency of chromosomal aberration. The values of mean chromosomal aberration frequency in treated group did not increase and showed non-significance ($p > 0.05$) when compared to negative control group. Concurrent positive control group showed mean chromosomal frequency of 11.0 with significant increase ($p < 0.05$) which demonstrated the sensitivity of the assay (Table 8).

The percent aberration per cell at all tested concentrations of beta NMN was found to be comparable to those observed in the vehicle control group. The percent aberration per cell in vehicle control group was 0.60 % while the test item showed 0.70 0.50 and 0.70 % at the dose levels of 2000, 1000 and 500 mg per kg of body weight respectively. The reversal group of highest dose (2000 mg/kg body weight) showed 0.50 % aberration per cell. The values of % aberrant cell in treated group did not increase and showed non significance compared to negative control group. Concurrent positive control group showed 22.0 percent aberration per cell with significant increase that is P less than 0.05 demonstrating the sensitivity of the assay (Table 8).

NAM inhibits the activity of PARPs, which function in recognizing and inducing repair of DNA strand breaks. Therefore, suppression of PARP activity raises reasonable concerns on loss of DNA integrity or enhanced sister chromatid exchange (SCE) (Schultz et al., 2003), which may causatively be associated with certain types of cancers (Cefle et al.,2006). For this reason, the carcinogenic and co-carcinogenic effects of NAM have been the focus of early studies. Hence both carcinogenicity or mutagenic activity of NMN was assessed. This is the first study to evaluate NMN for mutagenicity, if any, via in vivo mammalian bone marrow chromosomal aberration test.

The results obtained demonstrated that repeated oral administration of NMN appears to be safe and did not promote toxic effects as seen from bodyweight change, food and water consumption, feed conversion efficiency, biochemical and blood parameters as well as organ toxicity and histological examinations of main organs. NOAEL was estimated to be $\geq$ 800 mg/kg/d over a sub-chronic (90-day) treatment period.

The study highlights safety profile of NMN in animals and is suggestive of further safety studies in humans.

Conclusion

Nicotinamide adenine dinucleotide (NAD+) is an essential molecule involved in a myriad of cellular processes. However, NAD+ levels progressively decline with age, which is associated with age-related diseases. Our cells synthesize NAD+ primarily through its precursor nicotinamide mononucleotide (NMN). Although NMN supports increased NAD+ levels, which mitigates age-related diseases in animals. Still, there is a lack of studies demonstrating safety profile of NMN. We evaluated the toxicology profile of NMN (Uthever ®, a registered version of NMN from Effepharm (Shanghai) Co. Ltd.), through the acute oral toxicity, repeated dose (90-day) rat toxicity, bacterial reverse mutation, in vitro mammalian chromosomal aberration and in vivo mammalian bone marrow chromosomal aberration.

This study is first of its kind that investigated the genotoxic effects of NMN via *in-vivo* mammalian bone marrow. Genotoxic effects of any agent on the germinal cells give information about transmissible genetic damage from one generation to another (Au and Hsu, 1980, Tripathi et al., 2013b). In the in vivo mammalian bone marrow chromosomal aberration test, the female mice were exposed to different test item concentrations, vehicle and positive control during the conduct of main study. The mitotic index was within the control limit in the high, low and mid dose groups relative to controls. There was no increase in chromosomal aberrations in any treated group when compared to vehicle control group. There was no evidence for a statistically significant increase over the vehicle control values in the number of cells with

Table 8
Summary of Results-In vivo Chromosomal Aberration Assay in Swiss albino mice.

	Dose (mg/kg Body Weight)						
	G1	G2	G3	G4	G5	G6	G7
	Untreated	Vehicle control [a]	NMN 500	NMN 1000	NMN 2000	Positive control [b]	NMN 2000
Total metaphases scored	1000	1000	1000	1000	1000	250	1000
Total aberrations	10	6	7	5	7	55	5
Including gaps	10	7	8	6	8	64	7
Excluding gaps	10	6	7	5	7	55	5
Mean aberrations	2.00	1.20	1.40	1.00	1.40	11	1.00
Aberrations per cell(%)	1.0	0.6	0.7	0.5	0.7	22.0	0.5

Table 8 shows results for In vivo Chromosomal Aberration Assay in Swiss albino mice for untreated, vehicle control, Positive control and different concentrations of NMN. The table presents total metaphases, total aberrations, percentage of cells with structural aberrations.

[a] : Vehicle control = distilled water.

[b] : Positive control = 40 mg/kg methyl methane sulphonate.

chromosome aberrations following treatment. All vehicle control values were within historical control ranges and positive controls induced significant increases in the main chromosomal aberration frequency ($p < 0.05$). Under the conditions of this study it was concluded that NMN is non clastogenic at and up to 2,000 mg/kg body weight in all the animals tested.

In the acute oral toxicity study conducted, it was found that the acute oral LD50 of NMN was greater than 2000 mg/kg body weight with 5000 mg/kg body weight as LD50 cut-off value and is classified under "Category 5 or Unclassified" as per Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Based on the acute toxicity data of Utherver™, a dose ranging from low (200 mg/kg bw), medium (400 mg/kg bw) and high (800 mg/kg bw) dose was selected for 90-day repeated dose toxicity study. The results of this Repeated dose toxicity study helped to determine the No Observed Adverse Effect Level (NOAEL) of NMN. The NOAEL was considered to be NLT 800 mg beta NMN/kg body weight in rat under the experimental conditions. No deleterious changes in food and water consumption, body weight, organ weight, hematological, biochemical, oxidative stress and histopathological parameters were observed. In the bacterial reverse mutation test conducted, it was concluded that NMN is non-mutagenic at and up to 5000 μg/plate in all the strains of *Salmonella typhimurium* tested. The in vitro mammalian chromosomal aberration test determined that NMN is non-cytotoxic and non-clastogenic at and up to 2000 μg/ml in all the treatment groups tested. In the in vivo mammalian bone marrow chromosomal aberration test, the female mice were exposed to different test item concentrations, vehicle and positive control during the conduct of main study. The mitotic index was within the control limit in the high, low and mid dose groups relative to controls. There was no increase in chromosomal aberrations in any treated group when compared to vehicle control group. There was no evidence for a statistically significant increase over the vehicle control values in the number of cells with chromosome aberrations following treatment. All vehicle control values were within historical control ranges and positive controls induced significant increases in the main chromosomal aberration frequency (p less than 0.05). Under the conditions of this study it was concluded that NMN is non clastogenic at and up to 2,000 mg/kg body weight in all the animals tested.

The studies have provided the confidence that NMN could be explored safely further in humans for both acute and long term usage.

Funding

The authors(s) did not receive financial support for the research, authorship, and/or publication of this article.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crtox.2024.100171.

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