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## Safety of a novel oxygen-coordinated niacin-bound chromium(III) complex (NBC): I. Two-generation reproduction toxicity study

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## ABSTRACT

The objective of this study was to evaluate the effects of a novel oxygen-coordinated niacin-bound chromium(III) complex (NBC) on the reproductive systems of male and female rats, the postnatal maturation and reproductive capacity of their offspring, and possible cumulative effects through multiple generations. Sprague–Dawley rats were maintained on feed containing NBC at dose levels of 0, 4, 15, or 60 ppm for 10 weeks prior to mating, during mating, and, for females through gestation and lactation, across two generations. For the parents (F<sub>0</sub> and F<sub>1</sub>) and the offspring (F<sub>1</sub> and F<sub>2a</sub>), reproductive parameters such as fertility and mating, gestation, parturition, litters, lactation, sexual maturity and development of offspring were assessed. Results from the current study indicated that dietary exposure of NBC to parental male and female rats of both (F<sub>0</sub> and F<sub>1</sub>) the generations during the pre-mating and mating periods, for both sexes, and during gestation and lactation in case of female rats, did not cause any significant incidence of mortality or abnormal clinical signs. Compared to respective controls, NBC exposure did not affect reproductive performance as evaluated by sexual maturity, fertility and mating, gestation, parturition, litter properties, lactation and development of the offspring. Based on the findings of this study, the parental as well as the offspring no-observed-adverse-effect level for NBC was determined to be greater than 60 ppm in diet or equivalent to 7.80 and 8.31 mg/kg body weight/day in male and female rats, respectively.

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### 1. Introduction

A number of research has indicated that chromium(III) plays an important role in normal protein, fat and carbohydrate metabolism, as well as improves insulin sensitivity [1–9]. However, this traditionally proposed role of chromium(III) as an essential micronutrient has been a matter of debate in recent years largely due to the lack of understanding of the underlying mechanism of action by chromium(III) [10–12]. Nonetheless, a myriad of research including *in vitro*, *in vivo* and clinical studies demonstrated the beneficial effects of a novel oxygen-coordinated niacin-bound chromium(III) complex (NBC) in attenuating insulin resistance and lowering plasma cholesterol levels, increasing cardioprotective potential and lean body mass [6,7,13–16]. Studies have also shown that NBC is useful in overcoming sucrose-induced hypertension and has been shown to lengthen the life span of rats [16–19]. NBC has been demonstrated to prevent the increases in protein

glycosylation and oxidative stress caused by the high levels of glucose in erythrocytes [20]. Furthermore, NBC has been shown to attenuate cytokine secretion and oxidative stress in monocyte cell cultures treated with high level of glucose and acetoacetate [21]. The effects of NBC on lipid peroxidation, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin-6 (IL-6), C-reactive protein (CRP), glycated hemoglobin, triglycerides, and cholesterol levels have been examined in blood of streptozotocin (STZ)-induced diabetic rats [22]. The results from this study indicated that NBC was able to lower blood levels of pro-inflammatory cytokines, oxidative stress, and lipids levels in diabetic rats suggesting that NBC may lower the risk of vascular inflammation in diabetes [22]. A recent study using the model of STZ-induced diabetic rats after ischemia-reperfusion injury has revealed that NBC enhances myocardial protection [23]. This cardioprotective effect is mediated by increased activation of AKT, AMP-activated protein kinase (AMPK) and endothelial nitric oxide synthase (eNOS) resulting in an enhanced translocation of the insulin-regulated glucose transporter (Glut-4) to the caveolar raft [23]. The major forms of chromium(III) dietary supplements include chromium(III) chloride (CrCl<sub>3</sub>), chromium(III) picolinate (CrPic), and NBC [16,24]. A substantial number of studies have devoted to evaluating the safety and efficacy of these chromium(III)

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supplements. Several lines of evidence indicated that not all forms of chromium(III) supplements are equally safe and efficacious. *In vitro* study has suggested that CrPic was genotoxic resulting in mutation at the hypoxanthine (guanine) phosphoribosyltransferase locus of the Chinese hamster ovary (CHO) cells [25,26]. CrPic also induced clastogenesis or chromosomal damage in CHO cells whereas at the same physiologic dose and assay conditions, neither CrCl<sub>3</sub> nor NBC incurred any clastogenic effect [27]. Since the clastogenic effect only occurred in CrPic and picolinic acid but not in CrCl<sub>3</sub>, NBC or nicotinic acid, it was concluded that the observed clastogenicity was induced by picolinic acid and not by chromium *per se* [28]. However, several clinical cases have linked CrPic to nephrotoxicity resulting in renal failure [29,30]. A recent study by Nguyen et al. revealed that several formulations of chromium supplements, particularly CrPic, were able to undergo biological oxidation under *in vitro* conditions wherein chromium(III) was converted to chromium(VI) [11]. This may explained the observed genotoxic effects of CrPic. On the other hand, numerous studies on the safety of NBC have generally shown that there is no toxic effect associated with NBC [31,32]. The Nguyen et al. study indicated that not all formulations of chromium(III) complex had the same reactivity under biologically relevant conditions such as artificial digestion systems, blood and its components, cell culture media, and cultured intact L6 rat skeletal muscle cells [11].

In this regard, a comprehensive pharmacotoxicology rodent study has been conducted to evaluate the safety of NBC [31]. A battery of toxicology studies including acute oral, acute dermal, primary dermal irritation and primary eye irritation toxicities of NBC indicated a broad spectrum of safety for NBC [31]. Specifically, the acute oral LD<sub>50</sub> of NBC was found to be greater than 5000 mg/kg in both male and female Sprague–Dawley rats. No changes in body weight or adverse effects were observed following necropsy. The acute dermal LD<sub>50</sub> of NBC was found to be >2000 mg/kg [31]. The primary skin irritation test was conducted with NBC on New Zealand Albino rabbits. NBC was classified as slightly irritating. The primary eye irritation test was conducted with NBC on rabbits. NBC was classified as practically non-irritating to the eye. Ames bacterial reverse mutation assay and mouse lymphoma tests were also conducted. NBC did not induce mutagenic effects in the bacterial reverse mutation test in five *Salmonella typhimurium* strains (TA1535, TA98, TA100, TA97a and TA102), either with or without metabolic activation. Similarly, NBC did not induce mutagenic effects in the mammalian cell gene mutation test in L5178Y mouse lymphoma cells TK (+/–), either with or without metabolic activation. A dose-dependent 90-day subchronic toxicity study demonstrated no significant changes in selected organ weights individually and as percentages of body and brain weights [31]. NBC supplementation did not cause changes in hepatic lipid peroxidation or DNA fragmentation after 30, 60 or 90 days of treatment. Hematology, clinical chemistry and histopathological evaluations did not show any adverse effects in organs tested [31]. A 52-week long-term NBC safety study was investigated by orally administering either 0 or 25 ppm or the human equivalency dose of 1000 µg elemental chromium(III) as NBC per day for 52 consecutive weeks to male and female Sprague–Dawley rats [32]. Animals of each group and each gender were sacrificed on 26, 39, or 52 weeks of treatment. Body weight, physical and ocular health, feed and water intake, selected organ weights as such and as a percentage of liver and brain weight, hepatic lipid peroxidation and DNA fragmentation, hematology and clinical chemistry, and histopathological evaluations were conducted. At 26, 39, or 52 weeks of treatment, body weight gain was significantly reduced by 7.7%, 8.1% and 14.9% in male rats, and 5.5%, 11.4% and 9.6% in female rats, respectively, in the NBC treatment groups [32]. No significant changes were observed in hepatic lipid peroxidation and DNA fragmentation, hematology and clinical chemistry, and histopathological

evaluation between control and NBC groups at these time points [32].

The objective of the present two-generation reproductive toxicity study was to evaluate the effects of NBC on the reproductive systems of male and female rats, the postnatal maturation and reproductive capacity of their offspring, and possible cumulative effects through multiple generations.

## 2. Materials and methods

### 2.1. Protocol compliance

The study was performed in compliance with a standard study protocol approved by the Institutional Animal Ethics Committee (Protocol No. P/4489/RT-DT-R/06) based upon the United States Food and Drug Administration Redbook Guidelines for Reproduction Studies IV.C.9.a and Guidelines for Developmental Toxicity Studies IV.C.9.b., Feed Additive Safety and the Organization for Economic Co-operation and Development (OECD) principles of Good Laboratory Practice [33–35].

### 2.2. Animals

Male and female Sprague–Dawley rats, 7–9 weeks old, bred and reared at the animal breeding facility of INTOX Pvt. Ltd. India were used. The animals were maintained under controlled conditions in a room ventilated with 100% fresh and filtered air, with 10–15 air changes *per h*. The room temperature was maintained between 19 and 25 °C with relative humidity 30–70% and a 12 h light/dark cycle. The animals were allowed to acclimatize at least one week before the initiation of experiments with food and water available *ad libitum*.

### 2.3. Test compound

NBC is a unique, patented oxygen-coordinated niacin-bound chromium(III) complex commercially known as ChromeMate® CM-100 M (powder) (US Patents 4,923,855, 4,954,492 and 5,194,615). NBC [Lot#306013] was provided by the study sponsor of InterHealth Nutraceuticals, Benicia, CA, USA.

“Nutrilab” brand extruded rodent powdered feed manufactured by M/s Vetcare Pvt. Ltd., Bangalore, India, and tested for nutrients and contaminants, was provided *ad libitum* to the animals during the study period. NBC was mixed with powdered rodent diet to obtain the three concentration levels. Initially, a small volume of diet premix was prepared which was then mixed with remaining portion of diet in a mechanized ribbon blender for about 20 min to obtain desired homogeneity of the test article concentration in diet. The experimental diets were prepared once a week.

### 2.4. Treatment

Test article, NBC, for the study was provided by InterHealth Nutraceutical Inc. NBC was administered to rats orally, by dietary admixture. Since NBC is intended to be consumed by human beings up to a maximum dose of 4 mg of NBC/day, the highest dose level for this study was selected so as not to exceed 100 times the maximum recommended human dose, which has a dietary equivalent concentration of 60 ppm. A dose range study revealed no adverse effects of NBC on body weight, feed consumption, mating behavior, fertility, gestation or lactation in rat at dose level up to 60 ppm. The final experimental study design flow chart is summarized in Fig. 1. Sprague–Dawley rats (30/group/sex) were randomly divided into one control and three treatment groups (low, mid and high). The treatment groups of the F<sub>0</sub> parental generation received feed

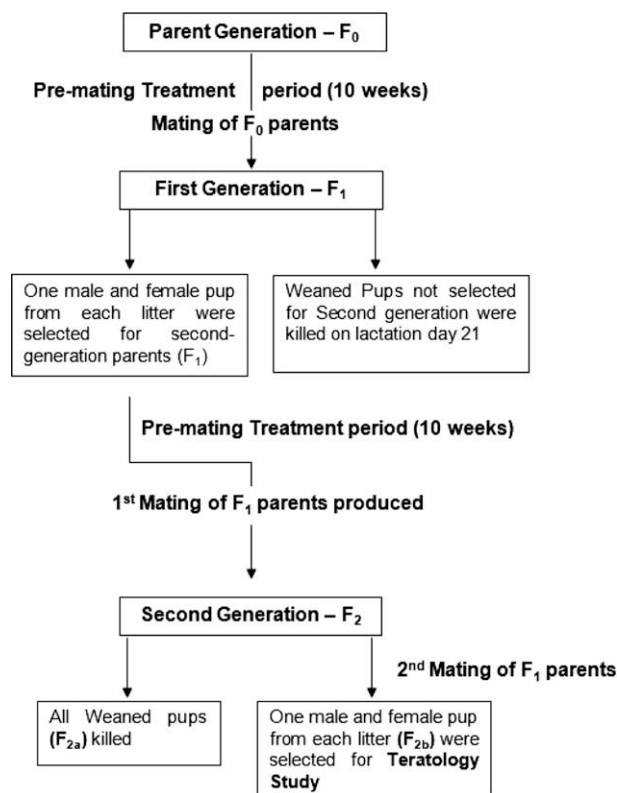


Fig. 1. Flow chart for two-generation reproduction toxicity study.

containing 4, 15, or 60 ppm NBC for a period of 10 weeks before mating, throughout mating, and continued until their termination. The exposure was continued through the next generations, up to completion of developmental toxicity study. Control group of animals were fed normal diet. The male and female rats of  $F_0$  generation from each dose group were mated and allowed to deliver normally. At weaning, one male and one female pup from each litter from control and treatment dose groups were selected for first filial ( $F_1$ ) generation. The selected  $F_1$  animals were exposed to NBC for 10 weeks before mating and then they were mated to produce second generation ( $F_{2a}$ ).

Animals were examined daily for signs of toxicity and mortality during the entire period of the study. Body weight and feed consumption of the animals from each group were recorded weekly. Feed consumption was calculated as g/rat/day. For both  $F_0$  and  $F_1$  parental animals reproductive parameters such as estrous cycle, female fertility index, gestation index, live-born index, mean litter size, sex ratio (at birth), number of stillbirths at day 0, number of live births at day 0, survival index, sperm count (epididymal and homogenization resistant testicular), sperm motility and sperm morphology were assessed. Pups from both generations were examined for survival, clinical signs, and their physical developmental landmarks such as unfolding of pinna (UP), hair growth (HG), teeth eruption (TE), eye opening (EYO) and ear opening (EO) were observed and recorded for appropriate lactation day to monitor postnatal growth. All pups, not selected for next generation, were sacrificed on lactation day 21, and subjected to necropsy.

After the mating and following confirmation of pregnancy status, males were euthanized with carbon dioxide and were subjected to necropsy and evaluation of sperm parameters such as sperm motility, cauda epididymis sperm count, testicular spermatid head count and sperm morphology. Sperm motility was assessed by observing percentile motile sperms and graded by

assigning a numerical score, based on the 6 point scale (see Table 1). The cauda epididymal sperm count was done by counting the sperms using a haemocytometer and was expressed as number of sperms per cauda epididymis and number of sperms per gram cauda epididymal weight.

The homogenization-resistant testicular spermatid head count was done by counting the spermatids using a hemocytometer and was expressed as number of (homogenization-resistant) spermatids per testis and number of spermatids per gram testis weight. The same cauda epididymis that was used for sperm motility and sperm count, was utilized for preparation of smears for sperm morphology evaluations which were then examined under  $40\times$  magnification. At least 200 sperms were counted per slide and abnormalities such as misshapen sperm-head, flagella, and sperm-head separation from flagella were recorded.

All females were killed after weaning. Organ weights were recorded at necropsy, and weighed organs from 10 randomly selected animals were subjected to histopathological examination. All animals, which died or were terminated during the study, were also subjected to necropsy and histopathological examination. Organ absolute weights were recorded and relative weights (% of body weights) were derived for both  $F_0$  and  $F_1$  parental animals. The organs weighed and examined included reproductive organs: epididymides (single and total), testes, prostate, ovaries, uterus, and seminal vesicles with Cowper's glands. In addition to the reproductive organs, other organs such as brain, pituitary gland, liver, kidneys, adrenals and spleen were weighed and examined for histological changes.

## 2.5. Statistical analysis

For statistical analysis, a litter was considered as the basic sampling unit. To analyze the various parameters, different and appropriate statistical methods were employed. For data on parental body weight and weight gain, feed intake, and organ weights Bartlett's test followed by ANOVA and Dunnett's test was employed with statistical significance at  $P < 0.05$ . Day 0 and absolute body weights were analyzed by paired  $t$ -test. Group differences in litter size were analyzed by Student  $t$ -test. Sex ratio was assessed by Chi-square test ( $2 \times 2$  contingency tables). Results of the statistical analysis were described as significantly higher (+)/lower (–) than control values at  $P < 0.05$ .

## 3. Results

### 3.1. Parental observations ( $F_0$ and $F_1$ )

#### 3.1.1. Food consumption and test compound dosage determination

Data on feed consumption by the parental male and female rats of both ( $F_0$  and  $F_1$ ) the generations during the pre-mating and mating periods, for both sexes, and during gestation and lactation in case of female rats, did not reveal any significant treatment related changes in the average daily feed intake by the male and female

Table 1

Sperm motility scale which was assessed by observing percentile motile sperms and graded by assigning a numerical score.

Motile sperms (%)	Description	Grade
0	No motility	0
<30	Poor motility, motility is sluggish	1
30–50	Fair motility, more than 30% but less than 50%	2
50–70	Good motility, progressive motile sperms	3
70–85	Very good motility, rapid progressive motile sperms	4
>85	Excellent motility, highly progressive motile sperms	5

**Table 2**  
Daily dose of NBC (mg/kg body weight/day) during pre-mating period.

NBC level in diet	F <sub>0</sub> males	F <sub>0</sub> females	F <sub>1</sub> males	F <sub>1</sub> females
Control (0 ppm)	0	0	0	0
Low (4 ppm)	0.38	0.49	0.43	0.66
Mid (15 ppm)	1.48	1.94	2.47	2.53
High (60 ppm)	5.88	8.24	9.71	9.83

rats compared to the respective control groups, across the different dose levels for each of the F<sub>0</sub> and F<sub>1</sub> generations and also when compared across these two generations. Based on feed intake, the resulting dose of NBC during pre-mating period for the highest dose groups F<sub>0</sub> male and female was calculated as 5.88 and 8.24 mg/kg/day, respectively, for F<sub>0</sub> generation, while the same was, respectively, 9.71 and 9.83 mg/kg/day in case of F<sub>1</sub> generation. The total daily dose of NBC for all groups is presented in Table 2.

### 3.1.2. Clinical signs and mortality

Compared to the respective control groups, across the different dose levels for each of the F<sub>0</sub> and F<sub>1</sub> generations and also when compared across these two generations, results of survival and clinical observations recorded for the parental male and female rats of both (F<sub>0</sub> and F<sub>1</sub>) the generations during the pre-mating and mating periods, for both sexes, and during gestation and lactation in case of female rats, did not reveal any remarkable incidence of mortality and abnormal clinical signs among the male and female rats exposed to NBC. All deaths and abnormal clinical signs observed in the rats during F<sub>0</sub> and F<sub>1</sub> generations, such as transient/reversible spells of emaciation, abdominal breathing, respiratory rales, hypoactivity, circling disorder and lacrimation, were considered to be incidental and not due to NBC feeding.

**Table 3**  
Summary of body weight (g) and body weight change (g) of F<sub>0</sub> and F<sub>1</sub> generation female rats during gestation.

NBC dose (ppm)	Body weight (g) on gestation days				% Gain (days 0–20)	Body weight change (g) during gestation days				
	0	7	14	20		0–7	7–14	14–20	0–20	
<b>F<sub>0</sub></b>										
0	263 ± 18	285 ± 20	312 ± 23	363 ± 30	38	22 ± 6	27 ± 9	51 ± 13	100 ± 16	
4	265 ± 17	287 ± 17	311 ± 16	364 ± 23	37	21 ± 6	25 ± 7	53 ± 12	98 ± 12	
15	261 ± 20	281 ± 22	312 ± 21	354 ± 29	36	20 ± 4	30 ± 9	42 ± 23	93 ± 22	
60	268 ± 23	290 ± 22	313 ± 26	357 ± 26	34	22 ± 7	23 ± 8	45 ± 13	90 ± 20	
<b>F<sub>1</sub></b>										
0	245 ± 17	265 ± 21	292 ± 22	346 ± 27	41	21 ± 7	27 ± 9	54 ± 11	101 ± 17	
4	243 ± 24	264 ± 26	288 ± 27	344 ± 37	41.4	21 ± 11	24 ± 8	56 ± 15	101 ± 22	
15	245 ± 20	263 ± 23	283 ± 26	335 ± 41	37	18 ± 6	20 ± 10	51 ± 25	89 ± 28	
60	255 ± 25	276 ± 25	299 ± 26	353 ± 31	39	21 ± 6	23 ± 10	54 ± 23	99 ± 28	

Each value is represented as mean ± SD (N ≥ 20/group). Treatment groups did not statistically differ from those of controls.

**Table 4**  
Summary of body weight (g) and body weight change (g) of F<sub>0</sub> and F<sub>1</sub> generation female rats during lactation.

NBC dose (ppm)	Body weight (g) on lactation days					% Gain (days 0–21)	Body weight change (g) during lactation days				
	0	4	7	14	21		0–4	4–7	7–14	14–21	0–21
<b>F<sub>0</sub></b>											
0	281 ± 21.8	278 ± 22.5	284 ± 21.5	275 ± 23.6	280 ± 27.1	−0.6	−3.0 ± 10.4	6.0 ± 8.8	−8.7 ± 12.1	4.1 ± 16.2	−1.6 ± 16.1
4	278 ± 15.8	277 ± 13.8	280 ± 18.4	273 ± 18.4	271 ± 14.7	−2.5	−1.2 ± 11.1	3.4 ± 11.4	−7.2 ± 11.1	−2.1 ± 15.0	−7.0 ± 16.0
15	273 ± 18.5	275 ± 20.2	275 ± 21.9	271 ± 20.0	262 ± 23.3	−4.1	3.4 ± 14.5	0.0 ± 8.5	−4.0 ± 12.3	−9.0 ± 13.9	−9.5 ± 20.5
60	279 ± 25.7	286 ± 23.9	286 ± 21.7	285 ± 24.4	282 ± 21.4	1.0	7.3 ± 15.1	−0.8 ± 9.7	−0.8 ± 11.8	−3.0 ± 19.9	2.7 ± 21.0
<b>F<sub>1</sub></b>											
0	269 ± 21.8	267 ± 20.6	273 ± 20.5	271 ± 25.6	282 ± 19.8	4.9	−2.4 ± 11.6	6.7 ± 10.0	−2.6 ± 19.5	11.4 ± 14.4	13.2 ± 13.5
4	267 ± 26.3	267 ± 28.4	276 ± 25.8	281 ± 26.6	282 ± 30.3	5.9	0.6 ± 14.5	8.4 ± 9.9	5.4 ± 16.0	1.3 ± 17.7	15.6 ± 24.7
15	265 ± 31.0	270 ± 28.3	275 ± 28.2	281 ± 30.4	286 ± 25.2	8.1	1.9 ± 12.4	5.7 ± 10.2	5.2 ± 15.1	2.2 ± 14.4	15.6 ± 16.5
60	274 ± 23.8	276 ± 24.5	277 ± 27.1	277 ± 29.0	284 ± 28.3	3.5	2.0 ± 15.2	1.0 ± 16.4	−0.5 ± 22.0	7.1 ± 18.0	9.6 ± 18.1

Each value is represented as mean ± SD (N ≥ 20/group). \*P < 0.05.

### 3.1.3. Body weights

The average body weight and body weight gains of the parental male and female rats of both the generations (F<sub>0</sub> and F<sub>1</sub>) during the pre-mating and mating periods, and during gestation and lactation of female rats, did not reveal any remarkable alterations which could be attributed to NBC exposure at any of the doses, when compared to the respective control groups, across the different dose levels for each of the F<sub>0</sub> and F<sub>1</sub> generations and also when compared across these two generations (Tables 3 and 4).

Although, other occasional instances of group mean values of treated animals differing from those of the respective control groups were noted, these were considered incidental or of no toxicological significance due either their lack of dose relation, their small magnitudes, or other procedural reasons unrelated to the treatment.

### 3.1.4. Mating, fertility and reproduction

Exposure of male and female rats, from both the F<sub>0</sub> and F<sub>1</sub> generations, to NBC at dose levels up to 60 ppm during pre-mating and mating periods and gestation period in females did not reveal any treatment related adverse effects on reproductive performance in terms of fertility and mating, gestation, parturition and the litters born. Similarly, the unaltered length and normalization of estrous cycles in treated females, mating performance as evidenced from unaltered indices of male fertility and female fertility, maintenance of normal gestation was evident from unaltered gestation length and gestation indices. Furthermore the pups born alive were unaffected, as evidenced from their live birth indices and did not reveal any treatment related adverse effects.

The values of male fertility indices for treatment groups in F<sub>0</sub> and F<sub>1</sub> generations did not differ significantly from those of the controls, and also compared well with the historical control data

at the test facility. The values of male fertility indices for treatment groups in F<sub>0</sub> generation in control, 4, 15 and 60 ppm groups were 90%, 100%, 80% and 92.2%, respectively. Similarly, these values for treatment groups in F<sub>1</sub> generation were 103%, 100% and 100% at the doses of 4, 15, and 60 ppm, respectively, while the value was 96% for the control group, respectively.

**3.1.4.1. Sperm evaluation.** For both the F<sub>0</sub> and F<sub>1</sub> generations exposed to NBC at dose level up to 60 ppm, evaluations of sperm parameters of male rats during pre-mating and mating period, and the period thereafter up to their termination, did not reveal any changes that could be attributed to the test article (Table 5). This was evident by virtue of the group mean values of motility of sperms in cauda epididymis, counts of sperms in cauda epididymis (absolute count and *per gram* of cauda weight), counts of homogenization resistant spermatids (absolute count and *per gram* of testis weight) *per testis*, and the morphological evaluations of the sperms by microscopy of stained smears. Although the sperm motility of F<sub>1</sub> parents compared to the F<sub>0</sub> parents was found to be slightly lower, it was not considered to be related to the NBC exposure, as the lowering was also observed in the concurrent control group of rats and the altered values were comparable to the historical control data.

### 3.1.5. Parental organ weights, necropsy and histopathology

At necropsy after the mating period (male rats) or the lactation period (female rats) the group mean values of absolute and relative weight (% of body weight and % of brain weight) of liver, kidneys, brain, spleen, adrenals, pituitary, testes, seminal vesicles (with cowper's glands), prostate, epididymides, ovaries and uterus, of male and/or female parental rats of F<sub>0</sub> generation and F<sub>1</sub> generation exposed to NBC at levels of 4, 15, and 60 ppm did not reveal any significant differences from the respective control group, which could be ascribed to NBC. Necropsy and histological examinations performed on the parents of the F<sub>0</sub> and F<sub>1</sub> generations, which died during the study or were terminated at end of the mating period (males) or the lactation period (females), did not reveal any incidence of gross and microscopic pathological alterations attributable to their exposure to NBC at dose levels of up to 60 ppm. All the gross and microscopic findings noted were considered to be incidental as the incidence was found to be comparable among the control group and the treatment groups, without any dose dependent trend.

## 3.2. Offspring observations (F<sub>1</sub> and F<sub>2a</sub>)

### 3.2.1. Offspring body weights

The data on average values of body weights of offspring of both the generations (F<sub>1</sub> and F<sub>2a</sub>) recorded on lactation days 0, 4, 7, 14

**Table 6**

Summary of sexual maturation of F<sub>2a</sub> offspring.

NBC dose (ppm)	Age (day) at sexual maturation	
	Male	Female
0	31.4 ± 2.58 (30)	57.4 ± 6.85 (30)
4	30.2 ± 2.76 (30)	54.4 ± 8.64 (30)
15	28.9 ± 1.80 (30)	51.7 ± 9.06 (29)
60	27.7 ± 1.42 (29)	50.8 ± 9.64 (27)

Each value is represented as mean ± SD (N). Treatment groups did not statistically differ from those of controls (*P* > 0.05).

and 21, did not reveal any alterations which could be attributed to exposure of their dams to NBC at levels up to 60 ppm, when compared to the respective control groups, across the different dose levels for each of the F<sub>0</sub> and F<sub>1</sub> generations and also when compared across these two generations.

### 3.2.2. Offspring clinical observations and mortality during lactation

Compared to the respective control groups of pups, across the different dose levels for each of the F<sub>1</sub> and F<sub>2a</sub> generations and also when compared across two generations, data on survival and clinical observations recorded for the offspring of both the generations (F<sub>1</sub> and F<sub>2a</sub>) during lactation period of 21 days did not reveal any remarkable differences. The observations included clinical abnormalities in pups, and the incidence of normal pups, pups found dead on lactation day 0 and thereafter, pups cannibalized by the dam on lactation day 0 and thereafter, and pups which were terminated in moribund state.

### 3.2.3. Litter observations

Comparison of the offspring data with respective control groups and also across the F<sub>1</sub> and F<sub>2a</sub> generations did not reveal any adverse effect on their litter sizes, the sex ratios of litters, the live birth indices and the viability indices of litters calculated for days 4, 7, 14 and 21 of lactation, following exposure of parental females to NBC at dose levels of 4, 15, and 60 ppm.

### 3.2.4. Offspring sexual maturation

The sexual maturation was measured only for F<sub>2a</sub> generation, in terms of age at which there is balanopreputial separation in males and vaginal opening in females (Table 6). Exposure to NBC at any of the dose levels did not affect the age of sexual maturity by the offspring belonging to the F<sub>2a</sub> generation. The group mean age at balanopreputial separation in male pups was 31.4 ± 2.58 days, 30.2 ± 2.76 days, 28.9 ± 1.80 days and 27.7 ± 1.42 days, respectively, for the control group and low, mid and high dose levels. Historical control value (Mean ± SD) for the same was 24.6 ± 2.7. The group mean age at vaginal opening in female pups was

**Table 5**

Effect of NBC on sperm parameters of F<sub>0</sub> and F<sub>1</sub> generation rats.

NBC dose (ppm)	Sperm motility	Cauda epididymal Sperm counts (×10 <sup>6</sup> )	Sperms/g cauda (×10 <sup>6</sup> )	Homogenization resistant testicular spermatid head count	
				Sperms (×10 <sup>6</sup> )	Sperms g testis (×10 <sup>6</sup> )
<i>F<sub>0</sub> generation</i>					
Control (0 ppm)	3.97 ± 1.05	41.52 ± 15.80	405 ± 170	5.08 ± 1.22	3.69 ± 0.66
4 ppm	4.26 ± 0.66	42.27 ± 16.26	422 ± 159	5.69 ± 1.15	3.93 ± 0.78
15 ppm	4.07 ± 0.94	41.96 ± 13.10	407 ± 82	5.75 ± 1.14	4.09 ± 0.79
60 ppm	4.33 ± 0.62	44.48 ± 16.80	406 ± 102	5.94 ± 1.11	4.05 ± 0.72
<i>F<sub>1</sub> generation</i>					
Control (0 ppm)	3.76 ± 0.78	36.61 ± 7.61	383 ± 62	6.74 ± 1.35	4.60 ± 1.01
4 ppm	3.64 ± 0.99	40.43 ± 9.10	449 ± 107	6.61 ± 1.33	4.61 ± 0.95
15 ppm	3.73 ± 1.04	42.36 ± 17.48	426 ± 135	6.12 ± 1.27	4.33 ± 0.90
60 ppm	3.69 ± 0.84	38.77 ± 14.75	386 ± 100	6.06 ± 1.52	4.18 ± 1.11

Sperm motility was graded on a scale of 0–5 (0 being no motility and 5 as excellent motility and highly progressive motile). *N* ≥ 27/group.

**Table 7**  
Summary of physical developmental landmarks of F<sub>1</sub> and F<sub>2a</sub>.

NBC dose (ppm)	UP	HG	TE	EYO	EO
<b>F<sub>1</sub></b>					
0	1.9 ± 0.5	3.4 ± 0.5	9.2 ± 0.8	13.6 ± 0.4	18.9 ± 0.7
4	1.8 ± 0.4	3.3 ± 0.5	9.4 ± 0.9	13.7 ± 0.4	18.7 ± 1.2
15	1.8 ± 0.5	3.7 ± 0.6	9.9 ± 1.0	14.0 ± 0.1	16.6 ± 1.3
60	1.7 ± 0.5	3.7 ± 1.1	9.3 ± 1.4	14.0 ± 0.1	17.9 ± 0.9
<b>F<sub>2a</sub></b>					
0	2.2 ± 0.6	3.7 ± 0.7	8.9 ± 1.0	13.8 ± 0.6	14.5 ± 0.8
4	1.9 ± 0.5	3.8 ± 0.5	9.6 ± 0.8	13.8 ± 0.4	14.5 ± 0.7
15	1.7 ± 0.6	3.6 ± 0.6	8.9 ± 0.7	13.9 ± 0.5	14.9 ± 1.1
60	1.9 ± 0.4	4.0 ± 0.5	10.1 ± 4.4	13.9 ± 0.9	15.1 ± 0.9

Each value is represented as mean ± SD ( $N \geq 16$ /group). Treatment groups did not statistically differ from those of controls ( $P > 0.05$ ). UP, unfolding of pinna; HG, hair growth; TE, teeth eruption; EYO, eye opening; EO, ear opening.

57.4 ± 6.85 days, 54.4 ± 8.64 days, 51.7 ± 9.06 days and 50.8 ± 9.64 days, respectively, for the control group and low, mid and high dose levels. Historical control value (Mean + SD) for the same was 49.7 ± 5.3. Although there was a trend for an inverse relationship between dose and sexual maturation in F<sub>2a</sub> generation, this trend was not statistically significant.

### 3.2.5. Offspring physical development endpoints

Exposure of the parental animals of both the F<sub>0</sub> and F<sub>1</sub> generation to NBC at 4, 15 and 60 ppm had no significant ( $P > 0.05$ ) adverse effect on the physical development of their litters during the period of lactation, which was evident by the unaltered period, in days, required by pups of F<sub>1</sub> and F<sub>2a</sub> generation to attain certain landmarks of physical development such as days required for unfolding of ear pinna, hair growth on the body, time (days) for eruption of teeth, opening of eyes and opening of ear, compared to the respective control groups (Table 7).

### 3.2.6. Offspring organ weights

Compared to the respective control group, exposure of the parental animals of both the F<sub>0</sub> and F<sub>1</sub> generation to NBC at dose levels of up to 60 ppm did not affect the organ weights of their

F<sub>1</sub> and F<sub>2a</sub> offspring. The group mean values of absolute and relative weights (% of body weights and % of brain weights) of brain, spleen and thymus of pups of F<sub>1</sub> and F<sub>2a</sub> generation did not significantly alter between the control and treatment groups (Table 8).

### 3.2.7. Offspring necropsy and histopathology changes

Necropsy performed on the offspring of the F<sub>1</sub> and F<sub>2a</sub> generations, on day 4 or at end of the lactation period, and histological examination of brain, thymus and spleen of pups euthanized at the end of lactation period did not reveal any incidence of gross or microscopic pathological alterations attributable to exposure of their parents to NBC at the dose levels of up to 60 ppm. All the gross and microscopic pathology findings encountered in this study were considered incidental as the incidence was found to be comparable among the control group and the treatment groups, without any dose dependent trend. Thus, NBC treatment did not cause any significant histopathological changes in any organ.

## 4. Discussion

The findings of this two-generation reproduction toxicity study demonstrate that exposure of male and female Sprague–Dawley rats to NBC at the dietary dose levels of 4, 15, and 60 ppm, (approximately corresponding to 0.5, 2 and 8 mg/kg/day, respectively) for over two generations was without any adverse effects on various parameters of reproductive performance such as growth, sexual maturity, fertility and mating, gestation, parturition, litter properties, lactation and development of their offspring. NBC, at these dose levels, did not induce any systemic toxicity in the parental rats and their offspring.

The present two-generation study serves as a better model for observing the influences of NBC on germ cell development, spermatogenesis, and sexual maturity. Results from the present study did not reveal any adverse effects of NBC on spermatogenesis at dose levels up to 60 ppm in F<sub>0</sub> and F<sub>1</sub> generation of male rats, respectively.

A broad spectrum of research investigations including *in vitro*, *in vivo* and clinical studies demonstrated the beneficial effects of NBC in promoting glucose-insulin sensitivity, lipid profile,

**Table 8**  
Summary of organ weights of F<sub>1</sub> and F<sub>2a</sub>.

NBC dose (ppm)	Relative weight (%)						
	% Body weight		% Brain weight				
	Brain	Spleen	Brain	Thymus	Spleen	Thymus	Spleen
<b>F<sub>1</sub> male</b>							
0	1.49 ± 0.16	0.23 ± 0.11	3.60 ± 0.89	0.40 ± 0.11	0.59 ± 0.36	11.14 ± 3.19	15.46 ± 6.88
4	1.54 ± 0.12	0.21 ± 0.07	3.84 ± 0.65	0.33 ± 0.09	0.50 ± 0.16	8.71 ± 2.50	13.56 ± 5.51
15	1.44 ± 0.21	0.19 ± 0.07	3.65 ± 0.75	0.40 ± 0.09	0.48 ± 0.16	11.65 ± 4.68	13.36 ± 3.60
60	1.53 ± 0.36	0.20 ± 0.09	3.79 ± 0.82	0.40 ± 0.07	0.49 ± 0.25	11.08 ± 2.31	13.35 ± 6.33
<b>F<sub>1</sub> female</b>							
0	1.49 ± 0.07	0.23 ± 0.10	3.81 ± 0.51	0.42 ± 0.14	0.59 ± 0.25	10.74 ± 3.18	15.60 ± 6.55
4	1.53 ± 0.03	0.22 ± 0.10	3.85 ± 0.46	0.35 ± 0.07	0.54 ± 0.21	9.17 ± 1.86	14.37 ± 6.57
15	1.43 ± 0.08	0.19 ± 0.08	3.84 ± 0.61	0.43 ± 0.07	0.50 ± 0.24	11.39 ± 1.58	13.25 ± 5.65
60	1.46 ± 0.32	0.16 ± 0.05	3.74 ± 1.10	0.45 ± 0.28	0.40 ± 0.10	12.79 ± 7.82	11.15 ± 3.15
<b>F<sub>2a</sub> male</b>							
0	1.44 ± 0.15	0.24 ± 0.11	3.22 ± 0.60	0.43 ± 0.10	0.50 ± 0.23	14.36 ± 5.97	16.81 ± 7.47
4	1.52 ± 0.05*	0.23 ± 0.07	4.03 ± 3.52	0.60 ± 0.57	0.60 ± 0.50	14.77 ± 2.44	15.40 ± 4.72
15	1.53 ± 0.07*	0.28 ± 0.24	2.98 ± 0.45	0.44 ± 0.07	0.50 ± 0.30	14.89 ± 3.12	18.04 ± 14.93
60	1.53 ± 0.05*	0.25 ± 0.10	3.02 ± 0.29	0.44 ± 0.06	0.49 ± 0.20	14.75 ± 2.06	16.41 ± 6.98
<b>F<sub>2a</sub> female</b>							
0	1.43 ± 0.09	0.21 ± 0.05	3.21 ± 0.64	0.43 ± 0.10	0.46 ± 0.12	13.87 ± 3.40	14.53 ± 3.81
4	1.46 ± 0.06	0.21 ± 0.06	2.98 ± 0.30	0.44 ± 0.07	0.43 ± 0.12	14.91 ± 2.55	14.65 ± 4.45
15	1.47 ± 0.05	0.21 ± 0.04	3.58 ± 2.55	0.52 ± 0.24	0.54 ± 0.58	15.65 ± 3.22	14.15 ± 2.64
60	1.49 ± 0.05	0.21 ± 0.06	3.02 ± 0.24	0.45 ± 0.05	0.42 ± 0.12	15.05 ± 2.31	14.01 ± 3.98

Each value is represented as mean ± SD ( $N \geq 14$ /group). Treatment groups did not statistically differ from those of controls ( $P > 0.05$ ).

cardioprotective ability and lean body mass [13–23]. In addition, a battery of toxicological studies including acute oral, acute dermal, primary dermal irritation and primary eye irritation indicated that NBC exhibited a broad spectrum of safety [31,32]. Furthermore, a number of human studies were conducted using NBC and no significant adverse effects were observed, which further substantiates the safety of NBC [13–16]. Supplementation of NBC in overweight people induced loss of fat mass and/or increased lean body mass. In a placebo controlled study with twenty overweight African American women over two months period with oral intake of 600 µg/day NBC with modest dietary and exercise regimen resulted in a significant fat loss without affecting muscle mass [13]. Blood chemistry analysis revealed no adverse events. Other human studies with daily doses ranging from 200 to 800 µg/day NBC exhibited similar results, and no significant adverse events were observed [14,15].

Overall, the results of two-generation reproductive toxicity in conjunction with earlier studies suggest that under the conditions of the study NBC is safe in male and female rats. The parental and offspring no-observed-adverse-effect level (NOAEL) in this two-generation reproduction toxicity study was found to exceed 60 ppm in diet. The equivalent dose in male and female rats was 7.80 and 8.31 mg/kg/day in male and female rats, respectively. These results, combined with previously conducted NBC clinical studies a decade ago, suggest the safe use of NBC at the recommended dose for human consumption.

## 5. Abbreviations

FDA	food and drug administration
NBC	oxygen-coordinated niacin-bound chromium(III)
NOAEL	no-observed-adverse-effect level
NOEL	no observed effect level
ADI	acceptable daily intake
OECD	organization for economic co-operation and development
DSHEA	dietary supplement health and education act
UP	unfolding of pinna
HG	hair growth
TE	teeth eruption
EYO	eye opening
EO	ear opening
CRP	C-reactive protein
Hb A(1)	hemoglobin A1
ANOVA	analysis of variance

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