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One-year chronic oral toxicity with combined reproduction toxicity study of a novel probiotic, *Bacillus coagulans*, as a food ingredient

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ABSTRACT

Some strains of *Bacillus coagulans* can survive extremes of heat, stomach acid and bile acids, to which commonly consumed probiotics are susceptible. A toxicological safety assessment was published in 2009 on a proprietary preparation of *B. coagulans* – GanedenBC³⁰™ – a novel probiotic. It was concluded that GanedenBC³⁰™ is safe for chronic human consumption based upon scientific procedures, supported by a safe history of use (Endres et al., 2009).

A one-year chronic oral toxicity study combined with a one-generation reproduction study was conducted to further investigate safety of long-term consumption. The one-year study of GanedenBC³⁰™ administered to male and female HsdBrlHan: Wistar rats in their diet showed no signs of toxicity at the highest dose tested. The conclusion from the reproduction toxicity study is that administration of GanedenBC³⁰™ in the diet caused no signs of toxicity in the parental generation (male or female) nor the F1 offspring. Using the lowest NOEL of 1948 mg/kg concluded at the end of the 1-year feeding study, a 100-fold safety factor, a test article concentration of 6.88×10^{10} CFU (colony forming units) per gram, and an average 70 kg human, it is determined that GanedenBC³⁰™ is safe for chronic consumption at up to 9.38×10^{10} CFUs per day.

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1. Introduction

Probiotics are defined as viable organisms (generally bacteria or yeast) that have demonstrated beneficial effects on the health of a host (Lee, 1999). Interest in probiotics for health promotion is at an all time high in more than 100 years they have been used (De Vecchi and Drago, 2006). The primary use of probiotics has been suggested for the prevention and/or mitigation of specific disorders such as irritable bowel syndrome (IBS), eczema, allergies, *Helicobacter pylori* infection, as well as for support of intestinal and immunological health (Tappenden and Deutsch, 2007; Quigley, 2007; Rastall et al., 2005; Hyronimus et al., 2000; Spiller, 2008; McFarland and Dublin, 2008; Lesbros-Pantoflickova et al., 2007; Ouwehand, 2007). Since 2009, the results of six clinical trials with GanedenBC³⁰™ have been published (Mandel et al., 2010; Kimmel et al., 2010; Dolin, 2009; Hun, 2009; Kalman et al., 2009; Baron 2009).

In general, probiotic organisms are sensitive to normal physiological conditions such as the very low pH of the stomach

and exposure to bile salts in the gastrointestinal tract. The viability of these bacteria can also be affected by manufacturing processes, storage, and shipping conditions. (Bezkorovainy, 2001; Ljungh and Wadstrom, 2006; Graff et al., 2008). Because *Bacillus coagulans* is a spore-forming bacterium, certain strains are able to survive the extremes of heat, acidity of the stomach, and bile acids, and thrive at physiological conditions (Patel et al., 2006; De Clerck et al., 2004; De Vecchi and Drago, 2006; Hyronimus et al., 2000; Katsutoshi et al., 2003). This increased hardiness, in comparison to typical probiotic organisms, results in a greater probability for survival through, and hence is able to transiently populate, the gastrointestinal tract (Adami and Cavazzoni, 1999). *B. coagulans*, when taken orally, has been demonstrated in a series of experimental studies to have beneficial effects on the gastrointestinal environment, stool frequency, and dermal appearance in animals and humans (Adami and Cavazzoni, 1999; Donskey et al., 2001; Katsutoshi et al., 2003). Because it lacks the ability to adhere to the intestinal epithelium, it is completely eliminated in four to five days unless chronic administration is maintained (Donskey et al., 2001). An unpublished study conducted by Goodner et al., reports the results of a PCR-based assay investigating GanedenBC³⁰™ for the potential *B. cereus* enterotoxin

Abbreviations: BC30 or GBI-30, GanedenBC³⁰.

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and haemolysin genes. The conclusion from the study was that GanedenBC^{30™} does not produce any sort of enterotoxin or haemolysin (Goodner, in press).

The history of *B. coagulans* dates back to 1915, when the discovery of curdled canned evaporated milk was first described by the Iowa Agricultural Experiment Station (Sarles and Hammer, 1932). In 1932, *B. coagulans* was first isolated and described by Horowitz-Wlassowa and Nowotelnow (Gandhi, 1994).

In 2009 we presented the first comprehensive safety assessment for the strain of *B. coagulans* known as GanedenBC^{30™} (*B. coagulans* GBI-30, 6086). That assessment was based upon not only a history of human exposure, but more importantly on the results of the following FDA, OECD, and GLP compliant toxicological studies: bacterial reverse mutation assay; chromosomal aberration and mouse micronucleus studies, skin and eye irritation study in the rabbit, acute oral toxicity (the limit test) study in the rat, and a 90-day subchronic repeat oral toxicity study in the rat (Endres et al., 2009). This paper presents the results of a US FDA Redbook compliant one-year chronic repeated oral toxicity study combined with a one-generation reproduction toxicity study in the rat. The study was approved by the Institutional Animal Care and Use Committee (IACUC) of Toxi-Coop Zrt., (Budapest, Hungary).

2. Materials and methods

2.1. Test product

The test product, GanedenBC^{30™}, supplied by Ganeden Biotech, Inc., (Mayfield Heights, OH, USA) is sold as a dietary ingredient for use in functional foods and dietary supplements. The organism is a gram-positive spore-forming rod that is aerobic to microaerophilic in nature. GanedenBC^{30™} is manufactured as a pure cell mass consisting solely of *B. coagulans*. The pure cell mass is either spray-dried with maltodextrin or freeze-dried and mixed with maltodextrin to achieve the desired concentration of 15×10^9 CFU/g for the finished product. For the purpose of these toxicological studies, pure, GanedenBC^{30™} (Lot# 90BC008–6.88 $\times 10^{10}$ CFU/g) was used.

The test article was mixed into standard rodent diet (Ssniff® SM R/M-Z+H, Ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) on a weight per weight basis and administered orally to animals at predetermined concentration levels. Fresh preparations of the mixture were prepared every three months. Concentration and homogeneity of the GanedenBC^{30™} cell mass were examined at each dose level and from each batch of the test diet. Control animals received only the standard rodent diet.

2.2. Chronic one-year oral toxicity study in rats

This study was conducted in accordance with procedures indicated by OECD Guideline for the Testing of Chemicals, No. 452 (12 May 1981) and US FDA Redbook 2000 standards IV.C.5.a., "Chronic Toxicity Studies with Rodents" (July 2007).

Male and female HsdBrlHan rats of Wistar origin (7–9 weeks old) were obtained from Toxi-Coop Zrt. (Budapest, Hungary). The animals were housed in individual cages, at a temperature of 22 ± 3 °C, a relative humidity of 30–70%, and a 12-h light-dark cycle. Animals were acclimated for 14 days prior to initiation of treatment.

Animals received Ssniff® SM R/M-Z + H complete diet for rats (Ssniff Spezialdiäten GmbH, Soest, Germany). Tap water was available *ad libitum*. Drinking water was analyzed once after the study and determined to be free of contaminants. An equal number of animals from each weight and sex group were randomized to the four experimental groups. Body weights of the animals at randomization were 175–217 g for males and 136–189 g for females. Each group consisted of 40 rats (20/sex). Animals were sorted according to body weight. An equal number of animals from each weight group were randomized to each of four experimental groups per sex.

A high target dose level of 2000 mg/kg bw/day was established with additional dosage levels of 0, 600 and 1200 mg/kg bw/day. These levels corresponded to a test article concentration in rat feed of 0, 10,000, 20,000 and 33,300 mg/kg. The highest target dose was chosen based on the results of previous toxicology studies showing a lack of toxicity of GanedenBC^{30™} (Endres et al., 2009). Dietary concentrations were not adjusted for body weight or food consumption during the study. All groups were fed diets containing the stipulated concentration of GanedenBC^{30™} for 52 to 53 weeks on a daily dosing schedule of a 7-day per week basis.

Observations for signs of morbidity and mortality were made twice daily, while general clinical observations were conducted once daily following treatment at approximately the same time each day. Detailed clinical observations were made on all animals outside the cage using a standard method both before the first exposure, and once a week thereafter. Observations focused on skin, fur, eyes, mucous

membranes, autonomic activity, circulatory and central nervous system, somato-motor activity, behavior pattern, tremors, convulsions, salivation, stool consistency, lethargy, sleep, changes in gait, posture and response to handling.

All animals were weighed on the day of randomization, on day 0 before the start of treatment, once weekly during the first 13 weeks of the study, and once every four weeks thereafter.

Food was weighed weekly, and the average food consumption per animal was calculated. Ophthalmological examination was performed before the study and at the end of the study. Clinical pathology examinations, including hematology and chemistry screens, were conducted in all animals at the end of the first 3 weeks, and at study months 3, 6, and 12. Urinalysis was performed the week prior to initiation of treatment, and at the end of study months 3, 6, and 12. Moribund animals were necropsied as soon as possible after observation. Any animals found dead were necropsied in the same manner as animals that were sacrificed at the end of the study. All surviving rats were sacrificed under Euthasol® anesthesia (Prodlab Pharma, Raamsdonksveer, Netherlands) and necropsied during week 53. External appearance of each animal was examined, followed by dissection of the cranial, thoracic and abdominal cavities to observe the appearance of tissues and organs. All abnormalities were recorded in detail. Organs were preserved in 10% formalin solution, while testes were preserved in Bouin's solution.

Measurements of organ weights were recorded, including the liver, brain, heart, thymus, spleen, kidneys, testes, epididymis, uterus, thyroid/parathyroid, adrenal glands and ovaries. Full histopathological exam was performed in the control (0 mg/kg bw/day) and high dose (2000 mg/kg bw/day) groups, and in animals found dead or moribund. Any observations of abnormalities in organs from the mid and low-dose groups resulted in histological examination of the relevant tissues.

Statistical analyses were performed to assess body weight and body weight gain, food consumption, hematology parameters, clinical chemistry parameters, urine parameters and organ weights. Evaluation of data was performed using SPSS PC+4.0.

2.3. One-generation reproductive toxicity study in rats

This study was conducted in accordance with procedures indicated by OECD Guideline for the Testing of Chemicals, No. 415 (26 May 1983) and US FDA Redbook 2000 standards IV.C.9.a., "Guidelines for Reproduction Studies" (July 2000).

The study was conducted in parallel to the one-year chronic oral toxicity study described above. Male and female HsdBrlHan rats of Wistar origin (7–9 weeks old) were obtained from Toxi-Coop Zrt. (Budapest, Hungary) and housed in the same environmental conditions described above. Animals were acclimated for 14 days prior to initiation of treatment.

For this study, parental animals were identified with numbers written in permanent marker on their tails following randomization into four dose groups (0 mg/kg bw/day (control), 600 mg/kg bw/day, 1200 mg/kg bw/day and 2000 mg/kg bw/day) as above. Each group consisted of 10 males and 20 females. Animals received the test article mixed into standard rodent diet and tap water *ad libitum*, as described above.

The test article-enriched diet, or control diet, in the appropriate concentration was dosed for ten weeks in all groups and during a three-week mating period. Male rats were dosed for 70 days before mating (corresponding to one complete spermatogenic cycle) and during the three-week mating period. Female rats were dosed with the test article mixed in the diet for ten weeks prior to mating, during the three-week mating period, throughout pregnancy and lactation and up to weaning of the F1 offspring.

The mating procedure consisted of one female being placed with a single male. Mating time was three hours daily in the morning for three weeks. The female remained with the same male until pregnancy occurred, or until the three weeks was over. Each morning a vaginal smear was prepared and stained with 1% aqueous methylene blue solution. The day of mating (identified by the presence of a vaginal plug or sperm on the vaginal smear) was considered as day 0 of the pregnancy. Sperm positive females were then separated from the males and housed singly.

Females that failed to mate within two estrous cycles were re-mated with proven males of the same test group. Pairs still failing to mate were evaluated to determine the cause of apparent infertility. This included additional opportunities to mate, microscopic examination of reproductive organs, and examination of the estrous cycle. Pregnant females were allowed to litter normally and rear progeny to the stage of weaning without standardization.

Observations for signs of morbidity and mortality, as well as detailed clinical observations, were made in the manner described above for the one-year study.

All animals were weighed on the day of randomization and on day 0 before the start of treatment. Male animals were weighed weekly until their necropsy. Female animals were weighed and evaluated weekly (on the same day) prior to and during mating, on gestation days 0, 7, 14 and 21, and on lactation days 0, 7, 14 and 21. Live pups were counted and weighed on the morning after birth and on days 4 and 7, and then weekly thereafter until termination of the study.

Food consumption was measured weekly during the pre-mating and mating periods. After parturition, and during lactation, food consumption was measured on the same day the litters were weighed.

Reproductive parameters of dams were assessed as follows: vaginal smears of all females were prepared daily during the pre-mating period and during mating. The smears were stained with 1% aqueous methylene blue solution and examined with a light microscope. Females were allowed to litter and rear their offspring. Dams were observed on the basis of whether or not they made a nest from provided bedding material and covered their newborns. Efficiency of nursing was observed by the presence of milk in the pups' stomachs. All observations were recorded.

The day of delivery of the F1 pups was defined as postnatal day 0. Two hours after delivery (following the first feeding), the offspring were individually identified by cutting off fingertips, were weighed, and sex was determined. Any observed abnormalities were recorded after identification.

All litters were checked daily for the number of viable and dead pups. Any dead pups were subjected to necropsy to identify the potential cause of death. F1 animals were weighed with an accuracy of 0.01 g up to the seventh postnatal day, and with an accuracy of 0.1 g until the 21st postnatal day. Weights were taken on days 0, 4, 7, 14 and 21. Pup development was assessed using surface-righting reflex, pinna detachment and eye opening.

Necropsy of the animals in these groups was conducted as follows: Males and non-mated females were sacrificed under anesthesia (as in the one-year study above) after the mating period and necropsied; animals failing to deliver up to gestation day 24, and dams with total litter death, were sacrificed and necropsied; dams with viable pups on lactation day 21 were necropsied, and the number of implantations in the uterus and the number of corpora lutea were counted. Any dead pups were examined to ascertain the cause of death, and were designated as live-born or stillborn. Offspring were sacrificed without necropsy following postnatal day 21.

Organ weights were measured and recorded for the parental animals, including the liver, brain, thymus, spleen, kidneys, testes, epididymis, uterus, adrenals and ovaries. Microscopic examinations were conducted on ovaries, uterus, cervix, vagina, testes, epididymis, seminal vesicles, prostate, coagulating gland and pituitary gland.

3. Results

3.1. Chronic one-year oral toxicity study in rats

The chronic one-year oral toxicity study on GanedenBC³⁰™ was performed to investigate for potential health hazards that are likely to arise as a result of repeated oral administration in the diet over a considerable part of the lifespan of HsdBrlHan rats of Wistar origin.

Analysis of the dietary intake of the rats and actual dose levels of GanedenBC³⁰™ in the various dose groups revealed the following: 600 mg/kg bw/day target dose group (males received a calculated mean daily intake of 585 mg/kg bw/day, females received 761 mg/kg bw/day); 1200 mg/kg bw/day target dose group (males: 1147 mg/kg bw/day, females: 1467 mg/kg bw/day); 2000 mg/kg bw/day target dose group (males: 1951 mg/kg bw/day, females: 2525 mg/kg bw/day).

No mortality occurred in any of the male groups (control, 600, 1200 and 2000 mg/kg bw/day) or the female group receiving 600 mg/kg bw/day of the test article. One female treated with 1200 mg/kg bw/day was found dead on day 137. Signs of irritability, stereotyped movement, abnormal gait, and eye and nasal drainage were observed on the preceding days. Gross pathology examination revealed autolysis of organs and tissues; therefore, histopathological examination was not feasible. The cause of death was attributed to an individual disorder. In the control group, one female was euthanized on day 104, while a female in the 2000 mg/kg bw/day group was euthanized on day 331. In the female in the control group, diffuse subacute dermatitis and cachexia were observed, along with an eventual decrease in body weight. In the female euthanized from the 2000 mg/kg bw/day group, an enlarged abdomen and skin scarring were noted. Pathological examination revealed generalized fibrosarcoma as the cause of the moribund condition. In accordance with historical control data of rats in the same age group, these alterations were considered to be individual occurrences, unrelated to consumption of GanedenBC³⁰™.

In terms of clinical observations over the course of the study, one male animal in the control group exhibited signs of irritability

for one day (day 315), while in the female animals of this group, one had decreased body tone and four had alopecia, while two showed signs of skin scarring. One male animal in the 600 mg/kg bw/day group exhibited periods of alopecia between days 46 and 153 of the study, interrupted with normal periods. In the females of this group, four exhibited alopecia and one animal showed signs of orbital swelling and eye drainage. In the 1200 mg/kg bw/day group, no clinical findings were noted in the males, while one female exhibited decreased body tone and another exhibited alopecia. No clinical signs were noted in males in the 2000 mg/kg bw/day group. In females of this group, one had sporadic body scarring, two showed a transient decrease in body tone, one exhibited a decreased grip tone, and another showed a decreased righting reflex. Overall, detailed clinical observations did not reveal any signs of toxicity related to chronic administration of the test article. The signs and symptoms occurring mainly in the females were considered individual alterations, as these signs are often seen in experimental rats and were not dose-related. In the two highest dose groups, the general physical condition and behavior of male animals were considered to be normal throughout the entire observation period.

Body weights of animals in all treatment groups remained similar to those of animals in the control group throughout the one-year treatment period. No effect of the test article was noted on body weight development.

Mean food consumption in all treatment groups was slightly below that of control animals during the entire observation period, with several weeks reaching statistical significance. The differences were likely a result of the taste of the mixed diet, were not found to be dose related, and were still well within the historical background range for the animals.

Ophthalmoscopic exam failed to detect any abnormalities in any animals pre-treatment. At the end of the study, hyperemic conjunctiva and sanguineous adnexa were found in one female in the 600 mg/kg bw/day treatment group, while corneal opacity was noted in a single female in the 2000 mg/kg bw/day group. None of these changes were treatment-related.

All hematological parameters were similar to the control values for both males and females in the 1200 mg/kg bw/day and 2000 mg/kg bw/day treatment groups, and for females in the 600 mg/kg bw/day group, when assessed at week three (Table 1). In male rats of the 600 mg/kg bw/day group, slight decreases were seen in mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCH) as compared to controls. However, no statistically significant differences were noted in hematological parameters in any of the groups at the end of the third month or at the end of the sixth month. At the end of the study (month 12), male animals in the 600 mg/kg bw/day group had a higher percentage of neutrophils, and a lower percentage of lymphocytes, while the hematocrit and partial thromboplastin time values were lower than control values. Male animals in the 1200 mg/kg bw/day group all exhibited values similar to controls, while in females of this group, white blood cell counts were slightly higher and prothrombin time was slightly lower than control animals. Male and female animals in the 2000 mg/kg bw/day group exhibited slight elevations in white blood cell counts compared to controls, while females were found to also have a slightly lower percentage of eosinophils than control animals. On the whole, no toxicologically relevant differences in hematological parameters in any treated groups were found at the end of week 3, or at the end of the 3rd, 6th and 12th months. The statistical differences seen in some parameters at the end of the 12th month were not considered to be toxicologically meaningful since they were of a low magnitude or of singular occurrence. Furthermore, all of these changes were within historical control ranges for this strain of rat.

Table 1
Summary of selected hematology and clinical chemistry data^a from the GanedenBC³⁰™ one-year repeated dose oral toxicity study in HsdBrlHan:Wistar rats.

Parameters	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1 (0–0)	2 (600–585)	3 (1200–1147)	4 (2000–1951)	1 (0–0)	2 (600–761)	3 (1200–1467)	4 (2000–2525)
Hematology								
<i>WBC</i> ($\times 10^9/L$)								
Week 3	9.76 ± 1.47	9.00 ± 1.53	9.48 ± 1.62	10.65 ± 3.28	8.07 ± 1.78	7.52 ± 1.70	7.43 ± 1.50	7.18 ± 1.48
Month 3	8.36 ± 2.11	7.91 ± 1.65	8.63 ± 1.59	8.34 ± 2.28	4.80 ± 1.05	5.18 ± 1.43	4.78 ± 1.00	4.90 ± 1.17
Month 6	7.84 ± 1.78	7.48 ± 1.56	7.74 ± 1.55	8.22 ± 1.73	4.81 ± 1.13	4.94 ± 1.42	4.55 ± 1.13	4.75 ± 1.69
Month 12	5.67 ± 1.22	5.45 ± 1.02	6.38 ± 1.32	7.14 ± 1.45**	3.79 ± 0.43	4.04 ± 0.74	4.50 ± 1.07*	4.73 ± 1.36*
<i>Neutrophils</i> (%)								
Week 3	13.74 ± 2.35	13.02 ± 3.15	12.25 ± 4.02	13.19 ± 4.64	10.06 ± 2.85	12.46 ± 3.61	11.65 ± 4.34	11.47 ± 4.55
Month 3	16.49 ± 4.32	18.58 ± 6.45	14.69 ± 3.05	17.16 ± 5.73	13.74 ± 3.99	15.49 ± 4.20	13.35 ± 4.17	15.41 ± 6.25
Month 6	16.95 ± 2.70	18.06 ± 6.25	15.97 ± 4.68	17.63 ± 4.69	15.23 ± 4.89	17.27 ± 6.27	17.57 ± 6.23	16.87 ± 8.43
Month 12	23.04 ± 4.65	28.25 ± 6.32**	24.12 ± 8.58	25.99 ± 8.31	24.21 ± 4.89	30.35 ± 8.35**	26.81 ± 6.04	23.99 ± 7.80
<i>Lymphocytes</i> (%)								
Week 3	82.97 ± 2.76	83.67 ± 3.37	84.62 ± 4.33	83.93 ± 5.00	86.57 ± 3.26	83.90 ± 4.19	84.76 ± 5.69	85.65 ± 4.85
Month 3	79.09 ± 4.61	76.91 ± 6.54	80.96 ± 3.62	78.35 ± 6.60	81.90 ± 4.48	80.12 ± 4.60	82.11 ± 4.83	80.86 ± 6.71
Month 6	78.26 ± 3.26	76.82 ± 6.79	78.79 ± 5.83	76.98 ± 4.93	80.05 ± 5.13	78.06 ± 6.17	77.29 ± 6.24	78.41 ± 9.51
Month 12	72.12 ± 5.16	66.16 ± 6.47**	70.73 ± 9.29	69.00 ± 9.01	69.23 ± 5.29	62.91 ± 8.32**	66.75 ± 5.63	70.08 ± 8.57
<i>Hematocrit</i> (L/L)								
Week 3	0.46 ± 0.01	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.01
Month 3	0.47 ± 0.02	0.66 ± 0.89	0.47 ± 0.03	0.47 ± 0.02	0.46 ± 0.01	0.46 ± 0.02	0.46 ± 0.02	0.46 ± 0.02
Month 6	0.47 ± 0.01	0.46 ± 0.02	0.47 ± 0.02	0.47 ± 0.02	0.46 ± 0.02	0.45 ± 0.02	0.46 ± 0.02	0.46 ± 0.02
Month 12	0.46 ± 0.02	0.44 ± 0.02*	0.46 ± 0.03	0.45 ± 0.03	0.44 ± 0.02	0.43 ± 0.03	0.44 ± 0.02	0.44 ± 0.02
<i>MCV</i> (fL)								
Week 3	52.46 ± 2.34	50.73 ± 1.94*	52.07 ± 2.19	51.89 ± 2.51	51.72 ± 2.79	51.61 ± 2.89	52.56 ± 2.87	51.74 ± 2.59
Month 3	49.65 ± 1.46	48.49 ± 2.41	49.65 ± 2.15	48.90 ± 2.44	51.42 ± 2.21	51.49 ± 2.00	52.19 ± 2.02	51.84 ± 1.92
Month 6	48.34 ± 1.57	46.97 ± 2.44	47.91 ± 2.02	47.84 ± 2.45	49.78 ± 2.34	50.14 ± 2.67	50.22 ± 2.00	50.29 ± 2.03
Month 12	48.37 ± 1.76	47.16 ± 2.59	48.64 ± 2.34	48.05 ± 2.87	50.51 ± 2.08	50.98 ± 2.78	50.75 ± 2.25	50.56 ± 1.85
<i>MCH</i> (pg)								
Week 3	18.27 ± 0.60	17.76 ± 0.61*	18.19 ± 0.65	18.02 ± 0.55	17.99 ± 0.82	18.01 ± 0.80	18.13 ± 0.71	18.00 ± 0.64
Month 3	17.33 ± 0.45	16.99 ± 0.69	17.36 ± 0.57	17.11 ± 0.63	17.73 ± 0.70	17.76 ± 0.66	17.92 ± 0.55	17.85 ± 0.52
Month 6	17.38 ± 0.43	16.95 ± 0.68	17.27 ± 0.54	17.26 ± 0.73	17.85 ± 0.67	18.00 ± 0.78	17.99 ± 0.63	18.00 ± 0.52
Month 12	17.68 ± 0.51	17.34 ± 0.77	17.79 ± 0.69	17.58 ± 0.84	18.33 ± 0.75	18.50 ± 0.87	18.45 ± 0.67	18.45 ± 0.59
<i>APTT</i> (sec)								
Week 3	20.05 ± 2.24	20.89 ± 1.61	21.13 ± 2.22	20.97 ± 2.22	20.56 ± 2.02	20.44 ± 1.96	19.71 ± 2.04	20.96 ± 2.03
Month 3	21.15 ± 2.59	21.26 ± 4.11	22.84 ± 3.05	21.38 ± 2.86	21.54 ± 2.22	21.73 ± 1.79	21.86 ± 1.70	22.66 ± 2.61
Month 6	22.00 ± 2.77	20.64 ± 2.83	21.39 ± 2.35	21.92 ± 3.08	22.99 ± 2.89	22.12 ± 1.58	22.39 ± 2.13	22.97 ± 1.83
Month 12	20.32 ± 2.38	18.43 ± 2.12*	19.18 ± 2.41	19.01 ± 2.52	18.93 ± 1.95	19.71 ± 1.83	19.44 ± 1.79	20.03 ± 1.25
<i>PT</i> (sec)								
Week 3	22.11 ± 1.93	23.00 ± 1.75	22.69 ± 2.79	22.34 ± 2.52	22.37 ± 2.02	21.45 ± 2.03	21.83 ± 2.26	22.99 ± 2.01
Month 3	21.55 ± 2.29	22.61 ± 4.92	22.88 ± 1.69	22.19 ± 2.15	22.19 ± 1.83	21.66 ± 1.86	21.73 ± 2.58	22.25 ± 2.24
Month 6	21.27 ± 3.42	20.69 ± 2.50	20.57 ± 2.57	21.40 ± 2.37	20.78 ± 2.04	20.34 ± 1.37	19.81 ± 1.70	20.76 ± 1.72
Month 12	25.26 ± 1.60	24.92 ± 1.80	24.07 ± 2.59	24.31 ± 2.14	22.12 ± 1.94	21.74 ± 2.24	20.47 ± 1.25**	21.38 ± 1.20
Chemistry								
<i>Glucose</i> (mmol/l)								
Week 3	5.99 ± 1.03	6.17 ± 1.05	5.92 ± 0.82	6.16 ± 0.95	5.56 ± 0.92	5.19 ± 0.94	5.63 ± 0.80	5.84 ± 0.85
Month 3	5.75 ± 0.92	5.69 ± 0.84	5.62 ± 0.76	5.43 ± 0.79	4.67 ± 0.72	4.61 ± 0.84	4.85 ± 0.49	4.98 ± 0.84
Month 6	7.12 ± 1.93	6.78 ± 0.79	6.98 ± 0.82	6.62 ± 0.96	5.86 ± 0.86	5.61 ± 0.84	5.96 ± 0.83	6.17 ± 1.12
Month 12	6.20 ± 0.60	5.66 ± 0.50**	5.79 ± 0.53*	5.32 ± 0.40**	5.13 ± 0.75	4.88 ± 0.70	4.99 ± 0.69	4.73 ± 0.57
<i>T. Bil</i> ($\mu\text{mol/l}$)								
Week 3	1.92 ± 0.31	1.90 ± 0.36	1.92 ± 0.38	1.86 ± 0.34	2.51 ± 0.50	2.47 ± 0.52	2.37 ± 0.53	2.58 ± 0.51
Month 3	3.17 ± 0.31	4.63 ± 6.16	3.20 ± 0.40	3.20 ± 0.56	3.54 ± 0.46	3.69 ± 0.34	3.72 ± 0.43	3.67 ± 0.61
Month 6	2.16 ± 0.34	2.15 ± 0.35	2.11 ± 0.47	2.19 ± 0.43	2.64 ± 0.65	2.77 ± 0.61	2.78 ± 0.66	3.00 ± 0.67
Month 12	1.88 ± 0.68	2.09 ± 0.44	2.25 ± 0.49	2.40 ± 0.63**	2.38 ± 1.14	2.68 ± 0.75	3.17 ± 0.99*	2.95 ± 1.04
<i>Phos</i> (mmol/l)								
Week 3	2.57 ± 0.19	2.40 ± 0.15**	2.47 ± 0.15	2.33 ± 0.19**	2.22 ± 0.28	2.07 ± 0.32	2.09 ± 0.25	2.07 ± 0.26
Month 3	2.15 ± 0.21	2.12 ± 0.23	2.05 ± 0.21	2.00 ± 0.15*	1.85 ± 0.30	1.76 ± 0.26	1.75 ± 0.37	1.66 ± 0.30
Month 6	1.85 ± 0.30	1.77 ± 0.21	1.82 ± 0.21	1.79 ± 0.21	1.60 ± 0.33	1.44 ± 0.28	1.41 ± 0.38	1.55 ± 0.35
Month 12	1.34 ± 0.20	1.42 ± 0.21	1.56 ± 0.20**	1.70 ± 0.20**	1.04 ± 0.19	1.27 ± 0.29**	1.48 ± 0.24**	1.60 ± 0.24**
<i>Na⁺</i> (mmol/l)								
Week 3	139.90 ± 1.21	140.15 ± 1.09	139.55 ± 1.05	139.60 ± 0.94	139.70 ± 1.49	139.40 ± 1.23	140.10 ± 1.17	140.20 ± 1.11
Month 3	141.75 ± 1.29	141.60 ± 1.14	141.75 ± 0.91	141.95 ± 0.60	140.75 ± 1.25	141.05 ± 1.15	141.25 ± 1.45	141.20 ± 1.67
Month 6	139.19 ± 1.30	139.43 ± 0.84	139.56 ± 1.19	140.08 ± 1.27	139.29 ± 2.02	138.67 ± 1.39	139.31 ± 1.46	139.49 ± 1.29
Month 12	142.14 ± 1.37	142.01 ± 1.64	141.94 ± 1.04	142.01 ± 0.80	141.71 ± 1.61	141.97 ± 1.65	141.25 ± 1.07	140.66 ± 1.60*
<i>Cl⁻</i> (mmol/l)								
Week 3	100.43 ± 1.46	100.84 ± 1.59	100.20 ± 1.14	100.59 ± 1.39	100.48 ± 1.74	100.64 ± 1.47	101.21 ± 1.72	101.24 ± 1.28
Month 3	101.40 ± 1.00	101.84 ± 1.27	101.51 ± 1.04	102.15 ± 1.12	101.67 ± 1.68	101.85 ± 1.43	102.57 ± 1.94	102.55 ± 1.66

Table 1 (continued)

Parameters	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1 (0–0)	2 (600–585)	3 (1200–1147)	4 (2000–1951)	1 (0–0)	2 (600–761)	3 (1200–1467)	4 (2000–2525)
Month 6	101.08 ± 1.08	101.74 ± 0.70 [*]	101.85 ± 0.90 [*]	101.85 ± 1.13 [*]	101.14 ± 1.80	100.70 ± 1.27	101.76 ± 1.69	101.19 ± 1.62
Month 12	104.02 ± 0.94	104.24 ± 1.01	103.64 ± 1.06	103.90 ± 1.09	103.54 ± 1.61	102.64 ± 2.22	103.31 ± 1.65	102.34 ± 1.51
<i>AST (U/l)</i>								
Week 3	99.92 ± 14.70	97.01 ± 16.63	99.92 ± 11.83	98.15 ± 13.91	102.69 ± 13.93	93.81 ± 6.80 [*]	91.05 ± 8.50 ^{**}	91.37 ± 11.66 ^{**}
Month 3	101.68 ± 15.80	101.49 ± 13.10	98.46 ± 12.04	98.57 ± 16.86	109.47 ± 18.55	99.45 ± 14.35 [*]	99.51 ± 11.55 [*]	100.31 ± 11.49 [*]
Month 6	116.00 ± 17.36	116.63 ± 21.88	114.83 ± 27.37	111.75 ± 25.28	110.60 ± 13.14	110.14 ± 16.41	114.52 ± 43.71	106.79 ± 26.02
Month 12	116.82 ± 25.81	115.38 ± 25.93	105.54 ± 20.95	114.09 ± 31.95	136.59 ± 40.90	129.79 ± 61.59	119.32 ± 39.74	120.03 ± 71.95 ^{**}
<i>ALT (U/l)</i>								
Week 3	47.83 ± 10.54	47.96 ± 12.22	48.40 ± 8.72	47.95 ± 9.92	45.11 ± 8.59	37.54 ± 5.25 ^{**}	37.63 ± 5.05 ^{**}	37.54 ± 7.18 ^{**}
Month 3	42.79 ± 9.40	44.59 ± 9.39	45.83 ± 10.76	44.02 ± 7.40	43.62 ± 7.30	41.25 ± 8.75	39.84 ± 5.47	46.53 ± 8.96
Month 6	62.01 ± 12.72	63.21 ± 16.57	65.88 ± 18.50	67.61 ± 17.28	59.97 ± 10.14	60.01 ± 11.50	59.18 ± 19.66	59.90 ± 19.04
Month 12	73.85 ± 26.76	70.63 ± 17.34	68.29 ± 20.73	69.60 ± 24.30	75.60 ± 16.26	77.30 ± 54.83	71.09 ± 29.26	73.85 ± 45.96
<i>GGT (U/l)</i>								
Week 3	1.75 ± 0.56	1.72 ± 0.38	1.77 ± 0.55	2.10 ± 0.50 [*]	2.36 ± 0.66	1.96 ± 0.56	2.26 ± 0.56	2.46 ± 0.69
Month 3	1.45 ± 0.65	1.83 ± 0.79	2.04 ± 0.75 [*]	2.06 ± 0.68 [*]	2.12 ± 0.69	2.03 ± 0.72	1.94 ± 0.82	2.18 ± 0.77
Month 6	1.64 ± 0.63	2.02 ± 0.76	2.14 ± 0.69 [*]	2.57 ± 0.70 ^{**}	1.96 ± 0.78	1.70 ± 0.84	2.04 ± 0.64	2.08 ± 0.38
Month 12	1.33 ± 0.91	1.71 ± 0.96	1.27 ± 0.76	1.40 ± 0.94	1.23 ± 0.89	1.03 ± 0.86	1.30 ± 0.81	1.39 ± 1.04
<i>Albumin (g/l)</i>								
Week 3	33.18 ± 0.95	32.99 ± 0.74	33.21 ± 1.03	33.12 ± 0.88	34.81 ± 1.31	34.97 ± 1.28	34.90 ± 0.92	35.52 ± 1.09
Month 3	33.63 ± 0.86	33.21 ± 0.84	33.32 ± 0.79	33.18 ± 0.96	34.90 ± 1.84	35.60 ± 1.15	35.17 ± 1.27	35.76 ± 1.61
Month 6	33.90 ± 1.08	33.37 ± 0.81	33.62 ± 0.87	33.53 ± 1.31	36.49 ± 1.75	36.29 ± 1.14	36.08 ± 1.21	37.19 ± 1.81
Month 12	34.01 ± 0.77	33.39 ± 0.79 [*]	33.37 ± 0.62 [*]	33.83 ± 0.89	37.17 ± 1.49	36.85 ± 1.57	36.99 ± 1.21	37.27 ± 1.46
<i>Protein (g/l)</i>								
Week 3	58.46 ± 2.38	57.93 ± 2.04	58.02 ± 2.31	58.08 ± 2.24	61.17 ± 3.02	61.58 ± 2.34	61.71 ± 2.19	62.86 ± 3.02
Month 3	60.43 ± 2.28	60.95 ± 1.78	60.91 ± 2.03	60.06 ± 2.50	62.47 ± 3.49	64.49 ± 2.60	64.34 ± 2.62	65.36 ± 3.67 ^{**}
Month 6	61.62 ± 2.89	61.06 ± 2.60	61.56 ± 1.74	61.01 ± 2.83	65.61 ± 3.98	66.05 ± 2.73	65.55 ± 2.38	67.67 ± 3.22
Month 12	62.43 ± 2.81	60.77 ± 1.89	61.53 ± 1.41	61.57 ± 2.30	66.88 ± 3.48	66.46 ± 3.65	67.31 ± 2.33	68.59 ± 2.95
<i>A/G ratio</i>								
Week 3	1.33 ± 0.09	1.34 ± 0.07	1.35 ± 0.08	1.33 ± 0.07	1.32 ± 0.08	1.31 ± 0.06	1.32 ± 0.09	1.29 ± 0.09
Month 3	1.25 ± 0.08	1.20 ± 0.08	1.22 ± 0.09	1.25 ± 0.11	1.27 ± 0.09	1.24 ± 0.08	1.25 ± 0.10	1.22 ± 0.08
Month 6	1.23 ± 0.08	1.21 ± 0.08	1.21 ± 0.07	1.23 ± 0.09	1.26 ± 0.09	1.22 ± 0.08	1.23 ± 0.08	1.22 ± 0.08
Month 12	1.20 ± 0.09	1.22 ± 0.06	1.19 ± 0.06	1.22 ± 0.06	1.26 ± 0.08	1.25 ± 0.08	1.22 ± 0.07	1.20 ± 0.09 [*]

Values are mean ± SD.

^{*} $p < 0.05$.

^{**} $p < 0.01$.

[‡] Data that is not shown was not statistically different between groups.

Clinical chemistry analyses revealed no clear test article related alterations (Table 1). Slight increases in gamma glutamate (GGT) activity in male animals at week 3 in the 2000 mg/kg bw/day group and at the 3rd and 6th months in the 1200 mg/kg and 2000 mg/kg bw/day groups disappeared at the end of the treatment period and remained within normal ranges throughout the study. Elevated phosphorus concentrations in all female treatment groups and in the 1200 mg/kg and 2000 mg/kg bw/day male groups at the end of the study were within historical control ranges. No histopathological changes were seen to substantiate their relevance. There were other sporadic statistical differences seen; however, none were considered to be of biological significance since they were within normal historical ranges for this strain of rat and no dose-response relationship was noted.

Urinalysis results were unremarkable for any of the treatment groups when compared to control values, when assessed pre-treatment, and at the end of the 3rd, 6th and 12th months. All values remained within the normal range throughout the study.

Necropsy results at the end of the study did not reveal any macroscopic changes that could be attributed to administration of GanedenBC^{30™}. In animals euthanized during the course of the study period, moribund conditions were caused by individual alterations. Pale liver and decreased testicular size occurred with similar incidence in control and treated males. These findings are also common in untreated rats and thus had no toxicological relevance. Findings in female genital organs included ovarian cysts, yellowish to dark red formations, thickened uterine wall

and horns, congestion and bleeding, and were observed in a very small number of animals in the control and treated groups; however, these are species-specific changes and also occur in untreated animals of this species of rat with similar age.

Organ weights assessed at the end of the treatment period were similar to the control values in all treatment groups. A slight statistically significant difference compared to control values was seen in mean liver weight of male rats in the 600 mg/kg bw/day group; however, this result was without any biological significance because there was no dose response and no correlating histopathology.

Histological examination did not reveal any test article-related or toxic lesions in the investigated organs. Several findings with similar incidence and severity occurred in the control and treated animals (Table 2), but were considered incidental, and were in accordance with historical control data. These included alveolar histiocytosis, mineral deposits, and pituitary adenomas. Furthermore, no morphological evidence of acute or subacute injury to the alimentary tract was observed, while examinations of the cardiovascular system, immune system, hematopoietic system, skeleton, muscular system and the central and peripheral nervous system were negative.

3.2. One-generation reproductive toxicity study in rats

This study was undertaken in order to obtain general information regarding the effects of GanedenBC^{30™} administration on

Table 2
Summary of histopathology findings from the GanedenBC³⁰™ one-year repeated dose oral toxicity study in HsdBrlHan:Wistar rats. The table contains only the list of organs with findings; however all organs and tissues stated in the study protocol were examined. Data is presented as the number of animals with findings/number of animals examined.

	Group number (mg/kg bw/day: targeted–actual) Males				Group number (mg/kg bw/day: targeted–actual) Females			
	1(0–0) n = 20	2 (600–585) n = 2	3 (1200–1147) n = 1	4 (2000–1951) n = 20	1 (0–0) n = 20	2 (600–761) n = 5	3 (1200–1467) n = 5	4 (2000–2525) n = 20
<i>Lungs</i>								
Emphysema	3/20	–	–	4/20	3/20	–	–	1/20
Alveolar Histiocytosis	6/20	–	–	3/20	4/20	–	–	1/20
Granuloma	0/20	–	–	0/20	1/20	–	–	0/20
Thymus								
Cyst	0/20	–	–	0/20	1/20	–	–	0/20
Skin								
Exudative dermatitis	0/20	–	–	0/20	1/20 ^b	–	–	1/20
<i>Kidney</i>								
Lympho-histiocytic infiltration	4/20	–	–	1/20	0/20	–	0/1	0/1
Pyelitis	1/20	–	–	1/20	1/20	–	0/1	0/1
Fibrosis	2/20	–	–	1/20	0/20	–	0/1	0/1
Mineral deposits	0/20	–	–	0/20	1/20	–	0/1	5/20
Cyst	0/20	–	–	0/20	0/20	–	1/1	0/1
<i>Liver</i>								
Normal	20/20	1/1 ^a	1/1 ^a	20/20	20/20	–	–	20/20
Sarcoma (metastasis)	0/20	–	–	0/20	0/20	–	–	1/20 ^{**}
<i>Pituitary</i>								
Adenoma	0/19	1/1	–	0/17	3/20	1/1	2/2	5/17
Spleen								
Sarcoma (metastasis)	0/20	–	–	0/20	0/20	–	–	1/20 ^{**}
<i>Testis</i>								
Decreased intensity of spermatogenesis	1/20	–	–	1/20	–	–	–	–
Ovary								
Cyst	–	–	–	–	2/20	–	–	0/20
Lack of corpora lutea	–	–	–	–	1/20	–	–	0/20
<i>Uterus</i>								
Sarcoma	–	–	–	–	0/20	–	–	1/1 ^{**}
Autolysis of organs	–	–	–	–	0/20	–	1/1 ^c	0/20
Dilation	–	–	–	–	3/20	2/5	0/3	2/20
Congestion	–	–	–	–	0/20	0/5	0/3	1/20
Hematoma	–	–	–	–	0/20	1/5	0/3	0/20
Endometritis	–	–	–	–	0/20	0/5	1/3	0/20
Estrus phase	–	–	–	–	1/20	4/5	2/3	0/20
Myoma	–	–	–	–	1/20	1/5	0/3	0/20

– = No data.

^a No abnormal findings correlating with necropsy observation.

^b Moribund animal (1 female in control group, 1 female in 2000 mg/kg bw/day group).

^c Dead animal (1 female in 1200 mg/kg bw/day group).

male and female reproductive performance in HsdBrlHan rats of Wistar origin.

Analysis of the dietary intake of the rats and mean dose levels of GanedenBC³⁰™ in the various dose groups revealed the following: 600 mg/kg bw/day target dose group (males received a calculated mean daily intake of 715 mg/kg bw/day, females received 1079 mg/kg bw/day); 1200 mg/kg bw/day target dose group (males: 1348 mg/kg bw/day, females: 2082 mg/kg bw/day); 2000 mg/kg bw/day target dose group (males: 2372 mg/kg bw/day, females: 3558 mg/kg bw/day). A further breakdown of the intake of GanedenBC³⁰™ in females of the parental generation revealed the following mean intakes in the different treatment groups during the pre-mating, gestation and lactation periods: 600 mg/kg bw/day target group (908 mg/kg bw/day during pre-mating period, 775 mg/kg bw/day during gestation, 1951 mg/kg bw/day during lactation); 1200 mg/kg bw/day target group (1769 mg/kg bw/day, 1593 mg/kg bw/day, and 3700 mg/kg bw/day); 2000 mg/kg bw/day target group (2983 mg/kg bw/day, 2596 mg/kg bw/day, and 6438 mg/kg bw/day).

Of the forty male and eighty female animals of the parental generation that composed the control and treatment groups, no

mortalities occurred during the entire observation period. Of the females, 17/20 became pregnant in the control group, whereas 15/20, 18/20 and 19/20 became pregnant in the 600 mg/kg, 1200 mg/kg and 2000 mg/kg bw/day treatment groups, respectively (Table 3). Of the pregnant animals, 17/17 delivered live born pups in the control group, whereas 14/15, 18/18 and 19/19 animals in the 600 mg/kg, 1200 mg/kg, and 2000 mg/kg bw/day groups, respectively, delivered live-born offspring.

Of the non-pregnant females in all groups (Table 3), follow-up revealed that all but one female from the control group was sperm-positive by vaginal smear. As included in results presented below, necropsy showed hydrometra in 1/3 non-pregnant females from the control group and 2/5 in the 600 mg/kg bw/day group. No other alterations were found by necropsy. Histopathology revealed uterine dilation in 2/5 non-pregnant females in the 600 mg/kg bw/day group, 1/2 in the 1200 mg/kg bw/day group and 1/1 in the 2000 mg/kg bw/day group. No other alterations were found by histopathology examination.

No clinical symptoms were noted for any groups of male animals and the physical condition and behavior was considered to be normal throughout the study. Two female animals were found

Table 3Summary of selected P(parents) data, delivery data and clinical observation of pups from the one-generation reproduction toxicity study on GanedenBC³⁰™.

	Group number (mg/kg bw/day targeted) Males				Group number (mg/kg bw/day targeted) Females			
	1 (0)	2 (600)	3 (1200)	4 (2000)	1 (0)	2 (600)	3 (1200)	4 (2000)
# of treated animals	10	10	10	10	20	20	20	20
# of animals that died	0	0	0	0	0	0	0	0
# of paired animals	10	10	10	10	20	20	20	20
# of mated animals	10	10	10	10	–	–	–	–
# of fertile animals	10	10	10	10	–	–	–	–
# of sperm positive animals	–	–	–	–	19	20	20	20
# of pregnant females	–	–	–	–	17	15	18	19
# of females with live pups	–	–	–	–	17	14	18	19
# of females not delivered	–	–	–	–	0	1	0	0
Duration of pregnancy (days)±SD	–	–	–	–	22.21 ± 0.54	22.16 ± 0.27	22.25 ± 0.36	22.26 ± 0.48
# of implantations	–	–	–	–	194	184	219	222
# of total births	–	–	–	–	179	164	184	206
No milk in stomach	–	–	–	–	4	4	1	3
Cold	–	–	–	–	1	4	3	1
Pale	–	–	–	–	0	0	1	0
Cyanotic	–	–	–	–	0	2	0	0
# of viable pups	–	–	–	–	177	160	182	206
On postnatal day 7	–	–	–	–	174	156	179	200
On postnatal day 14	–	–	–	–	174	149*	175	200
On postnatal day 21	–	–	–	–	174	148**	174	200
# of dead pups days 0–21	–	–	–	–	3	12**	8	6
On postnatal day 0–7	–	–	–	–	3	4	3	6
On postnatal day 7–14	–	–	–	–	0	7**	4*	0
On postnatal day 14–21	–	–	–	–	0	1	1	0
# of stillborns	–	–	–	–	2	4	2	0
# of extrauterine mortality	–	–	–	–	0	0	0	2
# of intrauterine mortality	–	–	–	–	17	24	37*	16
Live birth index (%)	–	–	–	–	99	98	99	100

* $p < 0.05$.** $p < 0.01$.

to have scarring. One animal was a part of the control group, while the second was a part of the 1200 mg/kg bw/day treatment group. All other female animals remained in normal physical condition, and had normal behavior, throughout the study period.

Body weights remained similar to control animals for males and females in all treatment groups throughout the study period. Furthermore, no differences in body weight or weight gain were noted between dams of the control and treatment groups.

No differences were seen in food consumption in males of all three treatment groups during the pre-mating and mating periods, or in females of all treatment groups during the pre-mating, gestation and lactation periods, when compared to controls.

No effects of the treatment were noted on the estrous cycle of any females; however, the estrous cycle was irregular in most animals of all groups for unknown reasons. Due to the low number of females with a regular cycle across all groups, correct evaluation of the cycle was not feasible. During the course of mating, the cycles became normal and no disturbances were noted in the mating process.

The mean duration of pregnancy was similar in all groups and no differences were seen in the percentage of dams that delivered, the percentage with viable fetuses and in live birth index. No effect of treatment was found in the number of implantations, number of total births, number of live-born, or stillborn pups. No stillborn pups were seen in the 2000 mg/kg bw/day treatment group.

No treatment-related effects were seen on reproductive performance of male or female animals, as the number and percentage of mated and fertile male animals, as well as the copulatory and fertility indices, were not affected. The number and percentage of non-pregnant females was highest in the 600 mg/kg bw/day treatment group and the percentage of pregnant animals was less than the control group. The percentage of pregnancies with live-born

offspring in this group were less than the control values, while the number of pregnancies not delivered exceeded control values; however, the pre-coital interval was similar in all groups.

Gross pathological examination did not reveal any treatment-related alterations. Pulmonary hemorrhages (1/10 animals in the 600 mg/kg, 2/10 in the 1200 mg/kg, and 1/10 in the 2000 mg/kg groups) and alveolar dilation (3/10, 5/10, 3/10 and 3/10 animals in the control and three treatment groups, respectively) were noted in some male animals across all groups, but were attributed to the exsanguination process and had no toxicological relevance. Hydro-metra was noted in three females in the 600 mg/kg bw/day group and one female animal in the control group, but is a frequent observation in experimental rats and has no toxicological significance.

Measurement of organ weights did not demonstrate any treatment-related effects. In males, there were slight differences from controls in the weights of the brain (600 mg/kg group) and testes (600 mg/kg and 1200 mg/kg groups) (Table 4). The weights of the adrenal glands were slightly below control values, while the weights of the kidneys were slightly higher (1200 mg/kg and 2000 mg/kg groups). In females, the weights of the adrenal glands (2000 mg/kg group) and ovaries (600 mg/kg group) slightly exceeded control values. However, all values were within historical control ranges. The differences in testes weight were not dose-related, as there were no such findings in the highest dose group.

Histopathological examination showed that the investigated reproductive organs in male animals of all groups were normal. In females, the ovaries had normal structure, characteristic for the species, age and phase of the active sexual cycle. The uterus, cervix and vagina all had normal structural characteristics. Some animals in the control and treatment groups showed dilatation of the uterine tubes, which is a normal occurrence in this strain of rat. In the

Table 4
Summary of organ weight data from one-generation reproduction toxicity study on GanedenBC^{30™}.

	Group number (mg/kg bw/day targeted) Males				Group number (mg/kg bw/day targeted) Females			
	1 (0)	2(600)	3 (1200)	4 (2000)	1 (0)	2 (600)	3 (1200)	4 (2000)
<i>Mean organ weights (g ± SD)</i>								
Body weight	406.6 ± 44.17	412.2 ± 46.87	380.1 ± 39.03	410.8 ± 37.68	270.2 ± 22.08	273.5 ± 31.36	276.7 ± 23.68	269.7 ± 21.41
Brain	2.29 ± 0.14	2.16 ± 0.10 [*]	2.18 ± 0.12	2.26 ± 0.13	1.84 ± 0.09	1.84 ± 0.11	1.83 ± 0.13	1.87 ± 0.10
Thymus	0.36 ± 0.04	0.36 ± 0.11	0.33 ± 0.09	0.34 ± 0.06	0.23 ± 0.25	0.16 ± 0.08	0.15 ± 0.08	0.15 ± 0.07
Spleen	0.83 ± 0.15	0.84 ± 0.09	0.79 ± 0.08	0.82 ± 0.08	0.66 ± 0.10	0.65 ± 0.11	0.63 ± 0.12	0.62 ± 0.12
Liver	12.78 ± 1.23	12.82 ± 1.49	11.75 ± 1.15	13.12 ± 1.54	12.15 ± 1.43	12.21 ± 2.53	12.10 ± 2.03	12.00 ± 1.63
Kidneys	2.52 ± 0.27	2.49 ± 0.27	2.43 ± 0.23	2.89 ± 0.50 [*]	1.87 ± 0.16	1.89 ± 0.31	1.97 ± 0.27	2.01 ± 0.29
Adrenals	0.083 ± 0.009	0.083 ± 0.009	0.072 ± 0.011 ₊	0.073 ± 0.009 [*]	0.090 ± 0.018	0.085 ± 0.013	0.096 ± 0.017	0.103 ± 0.019 [*]
Testes	3.54 ± 0.19	3.30 ± 0.35 ⁺	3.25 ± 0.14 ⁺	3.52 ± 0.27	–	–	–	–
Epididymides	1.60 ± 0.16	1.52 ± 0.19	1.48 ± 0.09	1.65 ± 0.19	–	–	–	–
Ovaries	–	–	–	–	0.116 ± 0.020	0.138 ± 0.023 [*]	0.133 ± 0.027	0.122 ± 0.026
Uterus	–	–	–	–	0.42 ± 0.11	0.45 ± 0.14	0.41 ± 0.08	0.45 ± 0.12
<i>Organ weight relative to body weight (% ± SD)</i>								
Brain	0.565 ± 0.031	0.527 ± 0.047	0.576 ± 0.045	0.553 ± 0.057	0.683 ± 0.054	0.679 ± 0.073	0.663 ± 0.039	0.694 ± 0.051
Thymus	0.089 ± 0.003	0.090 ± 0.039	0.086 ± 0.020	0.083 ± 0.016	0.088 ± 0.097	0.060 ± 0.036	0.055 ± 0.029	0.057 ± 0.031
Spleen	0.204 ± 0.032	0.206 ± 0.022	0.209 ± 0.021	0.200 ± 0.021	0.243 ± 0.032	0.238 ± 0.037	0.227 ± 0.043	0.231 ± 0.048
Liver	3.149 ± 0.126	3.113 ± 0.175	3.103 ± 0.260	3.189 ± 0.144	4.501 ± 0.432	4.445 ± 0.709	4.373 ± 0.600	4.461 ± 0.602
Kidneys	0.621 ± 0.036	0.605 ± 0.045	0.643 ± 0.054	0.700 ± 0.074 ^{**}	0.696 ± 0.058	0.688 ± 0.063	0.710 ± 0.052	0.748 ± 0.102
Adrenals	0.0205 ± 0.0029	0.0204 ± 0.0032	0.0188 ± 0.0019	0.0181 ± 0.0032	0.0334 ± 0.0056	0.0311 ± 0.0040	0.0349 ± 0.0064	0.0381 ± 0.0071 [*]
Testes	0.878 ± 0.091	0.806 ± 0.096	0.860 ± 0.074	0.860 ± 0.048	–	–	–	–
Epididymides	0.396 ± 0.053	0.371 ± 0.040	0.392 ± 0.034	0.403 ± 0.038	–	–	–	–
Ovaries	–	–	–	–	0.0434 ± 0.0082	0.0507 ± 0.0088	0.0483 ± 0.0094	0.0453 ± 0.0094
Uterus	–	–	–	–	0.156 ± 0.044	0.167 ± 0.062	0.150 ± 0.033	0.167 ± 0.046
<i>Organ weight relative to brain weight (% ± SD)</i>								
Body weight	17757.9 ± 1027.92	19105.4 ± 1769.26	17443.6 ± 1332.63	18245.2 ± 1770.97	14728 ± 1116.4	14880 ± 1483.6	15,125 ± 915.9	14,477 ± 1096.1
Thymus	15.79 ± 1.21	16.93 ± 6.22	14.92 ± 3.57	15.03 ± 2.22	12.73 ± 13.82	8.58 ± 4.33	8.17 ± 4.19	8.16 ± 4.14
Spleen	36.24 ± 5.78	39.12 ± 2.99	36.35 ± 3.37	36.45 ± 4.96	35.83 ± 5.23	35.16 ± 5.12	34.28 ± 6.55	33.32 ± 6.06
Liver	558.69 ± 31.40	593.79 ± 52.09	539.31 ± 36.28	583.07 ± 73.74	661.67 ± 71.97	663.30 ± 123.83	662.95 ± 111.16	643.84 ± 83.87
Kidneys	110.09 ± 6.86	115.33 ± 10.41	111.62 ± 6.63	128.53 ± 23.85	102.23 ± 9.42	102.37 ± 13.59	107.40 ± 10.90	107.97 ± 14.44
Adrenals	3.62 ± 0.43	3.85 ± 0.34	3.29 ± 0.40	3.26 ± 0.46	4.93 ± 0.96	4.61 ± 0.59	5.28 ± 0.96	5.51 ± 0.97
Testes	155.10 ± 10.59	152.78 ± 12.58	149.41 ± 9.06	156.55 ± 13.54	–	–	–	–
Epididymides	70.14 ± 7.92	70.39 ± 6.39	68.15 ± 4.08	73.54 ± 10.50	–	–	–	–
Ovaries	–	–	–	–	6.37 ± 1.19	7.50 ± 1.22 ^{**}	7.30 ± 1.47	6.52 ± 1.34
Uterus	–	–	–	–	22.83 ± 5.86	24.56 ± 7.60	22.57 ± 4.51	23.98 ± 5.94

Values are mean ± SD.

– = No data.

^{*} $p < 0.05$.^{**} $p < 0.01$.

sexually active animals the cortex contained primary, secondary and tertiary follicles and corpora lutea, indicating the active maturation of oocytes and ovulation.

In terms of the offspring, mortality was low across all treatment groups and control between postnatal days 0 and 21. However, there was a statistically significant slight increase in mortality observed for the 600 mg/kg treatment group compared to the control. The mean number of live pups was similar across all groups. The treatment groups showed no effects on the ratio of genders in the litters.

Clinical observation revealed no treatment-related effects in the offspring. Some common signs (including no milk in the stomach, pale, cold and cyanotic pups) were seen with similar incidence in the litters of all groups.

No treatment-related effects were seen with body weight gain and development, and the number of animals with negative responses on the surface-righting reflex and suckling ability assessments were comparable in all groups.

Furthermore, no treatment-related effects were noted on gross pathological examination in any of the offspring. Two stillborn pups from the control group were subjected to necropsy, which revealed autolyzed organs (1/2 pups), hydrops fetalis (1/2) and opened ductus Botalli (2/2). Four stillborn and one dead pup were subjected to necropsy in the 600 mg/kg treatment group. A patent ductus Botalli was seen in all stillborn pups in this dose group, while abdominal organs were autolyzed in the dead pup. In the

1200 mg/kg treatment group, two stillborn pups were found to have autolyzed organs, patent ductus Botalli and airless lungs, while in the 2000 mg/kg group, two live born pups were autopsied on day 0. No macroscopic findings were seen in one pup, while in the other, no milk was found in the stomach and necrotic lesions were found in the liver. None of these findings were deemed to have toxicological significance or attributable to administration of GanedenBC^{30™}.

4. Conclusion

The study was conducted as a continuation of the previously published safety assessment of GanedenBC^{30™} (Endres et al., 2009), a commercially available probiotic strain of *B. coagulans*.

A one-year chronic oral toxicity study combined with a one-generation reproduction study was conducted to further investigate safety with long-term chronic consumption. The study reported herein adds to the body of evidence in support of chronic consumption of GanedenBC^{30™} by humans. The conclusion from the one-year study is that GanedenBC^{30™} caused no signs of toxicity in male or female HsdBrlHan: Wistar rats after one year of diet-mixed administration. The NOEL was 1948 mg/kg bw/day for the males and 2525 mg/kg bw/day for the females – the highest dose tested. The conclusion from the reproduction toxicity study is that GanedenBC^{30™} caused no signs of toxicity on the parental generation (male or female) of the same strain of rat during the course of

the study with diet-mixed administration. The NOEL for the male rats is 2372 mg/kg bw/day and 3558 mg/kg bw/day for the females of the parental group. The NOEL for the reproductive performance for the males is 2372 mg/kg bw/day and 3558 mg/kg bw/day for females. The NOEL for the F1 offspring is 3558 mg/kg bw/day. In conclusion, to calculate the acceptable daily intake (ADI), the lowest NOEL from the study of 1948 mg/kg was used and a 100-fold safety factor was applied. Since the test article concentration was 6.88×10^{10} CFUs/g, using 70 kg as the mass for an average human, it is determined that GanedenBC³⁰™ is safe for chronic consumption at an accumulative daily intake of 9.38×10^{10} CFUs per day.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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