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Teratogenicity of 3-hydroxynorvaline in chicken and mouse embryos

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Summary. 3-Hydroxynorvaline (HNV; 2-amino-3-hydroxypentanoic acid), a microbial L-threonine analogue, is toxic to mammalian cells and displays antiviral properties. In view of this, we investigated the toxicity and/or potential teratogenicity of HNV in developing chicken and mouse embryos. HNV was administered to chicken embryos (*in ovo*; dose 75–300 μmole/egg; 48 h post-incubation) and pregnant Hanover NMRI mice (*per os*; total dose 900–1800 mg/kg body mass; gestation days 7–9). Control animals received sterile saline solutions. Harvested embryos (chicken embryos, 10 days post-incubation; mouse embryos; gestation day 18) were fixed in glutaraldehyde and stereomicroscopically inspected for signs of dysmorphogenesis. Body mass, body and toe length and mortality of chicken embryos, and the body mass and mortality of mouse embryos were recorded. HNV exposure significantly increased the incidence of embryotoxic (growth retardation, toxic mortality) and congenital defects in both chicken and mouse embryos. All the observed effects were dose-dependent. In conclusion, HNV is an embryotoxic and teratogenic compound, which caused significant developmental delay and congenital defects in developing chicken and mouse embryos.

Keywords: 3-Hydroxynorvaline – β-Hydroxynorvaline – Toxic amino acid – Teratogen – Dysmorphogenesis – Chicken embryo – Mouse embryo – Neural tube defects

Introduction

Numerous noxious and often highly toxic xenobiotics like mycotoxins and non-protein amino acids are produced by plants and microorganisms such as fungi and bacteria. This subsequently contaminates the food chain of man and domestic animals (Diaz, 2005; Dorne et al., 2005; Marasas et al., 2004; Speijers and Speijers, 2004; Van Egmont, 2004; Pohland, 1993; Kuiper-Goodman, 1991). Over the past four decades, hundreds of non-protein amino acids were isolated from plant extracts and growth cultures of microorganisms (Fowden et al., 1979; Pruess and Scannell, 1974; Rubinstein, 2000; Ravindranath, 2002; Wink, 2003; Bell, 2003). A significant number of these have potent antimetabolic properties in plants, microorganisms, experimental and domestic animals, and more importantly, in man.

3-Hydroxynorvaline (HNV) is a toxic microbial L-threonine analogue, with antiviral properties (Stocco and Clark, 1993; Green and Orme-Johnson, 1991; Pan and Elbein, 1990; Barile et al., 1989; Schwartz, 1988; Massare and Blough, 1987; Kumarasamy and Blough, 1984; Hortin and Boime, 1981; Kelley and Schlesinger, 1978; Christner et al., 1975). It was first synthesized in 1955 (Sunko and Kisic, 1955) from copper sulphate and glycine under alkaline conditions. Subsequently, Potgieter et al. (1976) obtained the 2S, 3R-isomer of HNV after catalytic hydrogenation of the toxic non-protein amino acid, 2S, 3R-2-amino-3-hydroxypent-4-ynoic acid (Rolfsin), which was purified from sclerotia of the fungus *Sclerotium rolfsii* (*Sacc.*). HNV apparently incorporate into cycloheptamycin, a microbial peptide (Godtfredsen et al., 1970), displaying potent antimicrobial activity. Two novel nucleosides were identified in unfractionated transfer RNA which was prepared from thermophilic bacteria and hyperthermophilic archaea and mesophilic archaea (i.e. *Thermodesulfobacterium commune*, *Pyrobaculum islandicum*, *Methanococcus vannielii*, etc). The nucleosides contain a residue of HNV in their structures (Reddy et al., 1992). The purification of free HNV from plants or microorganisms has not yet been reported.

Most, if not all, of the toxic effects of HNV can be ascribed to its incorporation into cellular proteins. HNV is a very close structural analogue of L-threonine. It can therefore readily substitute L-threonine in the primary structure of peptides and proteins and subsequently affect

a wide variety of molecular processes in exposed cells. HNV can be activated by the same aminoacyl-tRNA-synthase that activates L-threonine before its incorporation into peptides/proteins (Christner et al., 1975). Some of the toxic effects of HNV include miscleavage of pre-prolactin in rat pituitaries (Hortin and Boime, 1981), increased intracellular collagen degradation in human fetal lung fibroblasts (Barile et al., 1989) and the inhibition of O- and N-glycosylation of glycoproteins (Breuer et al., 2001; Docherty and Aronson, 1985). It has antiviral effects, inhibits herpes thymidine kinase and DNA-polymerase (Massare and Blough, 1987) and syncytia formation in HIV infected cells (Blough et al., 1986). HNV can also block steroidogenesis in rat adrenal cortex cells (Stocco and Clark, 1993; Green and Orme-Johnson, 1991). Since HNV has never been used in toxicological studies on animals, its potential toxicity and teratogenicity in animals is not known. The objective of this investigation was therefore to determine if HNV display teratogenic properties and can cause dysmorphogenesis in developing chicken and mouse embryos.

Materials and methods

Reagents

Propionaldehyde, copper (II) sulfate ($CuSO_4 \cdot 5H_2O$), glycine, KOH, thioacetamide, D,L-3-hydroxynorvaline (pure standard), glutaraldehyde and active charcoal were of the highest purity and purchased from Sigma Chemical Company. Dowex 50 X-8 was obtained from BDH. High quality fertilized eggs from inbred White Leghorn hens were obtained from the Department of Virology, Onderstepoort Veterinary Institute, Pretoria.

Chemical synthesis of 3-hydroxynorvaline

Diastereomeric 3-hydroxynorvaline was synthesized using the method of Sunko and Kisic (1955): Briefly, aldol condensation of propionic aldehyde (2.5 equivalents) with glycine was achieved by treating an alkaline solution (5 M NaOH) of copper glycinate (1 equivalent) under a nitrogen blanket (4 h; 4°C). The copper hydroxynorvalinate complex was then disproportionated by adding thioacetamide (1.5 equivalents). Precipitated copper sulphide was then removed by centrifugation ($2000 \times g$; 10 min) and subsequent filtration. Free, diastereomeric HNV was subsequently extracted and purified from the filtered, active carbon decolorized reaction mixture by cation exchange chromatography (Dowex 50 X-8; H^+). HNV was twice recrystallized in 80% methanol. The purity and identity of the synthetic product (>90% yield) was assessed by comparison of its molecular spectra (300 MHz ^1H and ^{13}C NMR spectroscopy; electrospray tandem mass spectrometry) to that of a crystalline standard from the Sigma Chemical Corporation.

Treatment of chicken embryos

This part of the study was approved by the Ethics Committee of the University of Pretoria: Pretoria, South Africa. Approximately 500 eggs were randomly divided into four groups. To prevent embryonic development, the eggs were stored at 12°C. Prior to dosage, all the eggs were simultaneously placed in an incubator to initiate embryonic development. Eggs were subsequently incubated in a Buckeye model 20 incubator (37.8°; 65% relative humidity). The shelves tilted automatically through 90° every 50 minutes. After 40 hours (13 somites, stage 11 of Hamburger and Hamilton, 1951), the blunt ends of the eggs were sterilized with 70% alcohol and a small hole (1 mm diameter) drilled through each egg shell, directly above the centre of the air sac. An aliquot (50 μL) of a filter-sterilized solution of diastereomeric HNV was injected into the air sac, so that the droplets came to rest on the porous membrane to which the developing embryo was connected. The experimental subgroups received an aliquot of 75 mM, 150 mM and 300 mM, respectively. Since the average volume of an egg was 50 ml, the final HNV concentration was approximately 75 μM, 150 μM and 300 μM, respectively. Control eggs received 50 μl of sterile saline. All eggs were resealed with paraffin wax and returned to the incubator for the full duration of the experiment.

Chicken embryos were sacrificed on the 10th day of development (stage 36 of normal development; Hamburger and Hamilton, 1951) and stereomicroscopically assessed for any gross anatomical abnormalities and mortality. After fixation in Allen's fluid, individual embryos was weighed, the total body length, the length of the beak and the length of the third toe measured.

In utero exposure of mouse embryos to HNV

Hanover NMRI females were used to study the effect of HNV on developing mouse embryos. This part of the study was approved by the Ethics Committee of the North-West University: Potchefstroom, South Africa. All animals were pathogen free and healthy and kept under controlled

Table 1. A summery of all defects observed in 10-day-old chicken embryos exposed to a single dose of HNV. The final HNV concentration was approximately 300 μM

Type of defect	Number of embryos	Percentage embryos	One-sided p-value
Encephalocele	1	0.8	0.2461
Spina bifida	2	1.5	0.1668
Incomplete closure of skull roofs	5	3.8	0.0608
Abnormal tail buds	3	2.3	0.1151
Total neural tube defects	*11*	*8.4*	*0.0099*
Omphalocele	2	1.5	0.1668
Total congenital defects	*13*	*9.9*	*0.0055*
Non-specific defects[1]	34	26.0	<0.0001
Total defects	*47*	*35.9*	*<0.0001*

[1] Growth retardation, subcutaneous hemorrhage, focumelias

conditions (ambient temperature: 21 ± 1°C; relative humidity; 55 ± 5%; a 12 hour light:dark cycle which changed at 0600 h and 1800 h). Air changes were kept constant (the total volume of air in the room was replaced 15–20 times per hour) and a light intensity of 350–400 lux was maintained 1 meter above floor level. Animals were fed a standard laboratory diet and had free access to food and water.

Virgin females were mated overnight with experienced males (1600 h–0800 h). The presence of vaginal plugs was accepted as confirmation that copulation was successful and these females were regarded as pregnant. The following 24 hours was designated as the first day of gestation. The experimental animals were treated orally with HNV (300, 450 or 600 mg/kg, respectively), while control animals received saline (0.2 ml) on days 7, 8 and 9 of gestation.

Animals were sacrificed by decapitation on day 18 of gestation. Embryos were removed, dried on filter paper and weighed before stereomicroscopic examination to detect gross anatomical abnormalities. Embryos were subsequently fixed in Todd's fixative for photographic purposes. All cases of mortality were noted.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). The Levene test for homogeneity of variances and the Shapiro-Wilk W-test were used to determine if the data was normally distributed. The Kruskal-Wallis non-parametric test was used to test for statistically significant differences. Fisher's exact test was used to analyze the relationship between concentration and death rates as well as the rates for the induction of neural tube defects. Differences were regarded as significant if $p \leq 0.05$.

Results

HNV (300 μM) induced a variety of neural tube defects in 10-day-old chicken embryos. These include encephalocele, spina bifida, incomplete closure of the skull roofs and abnormal tail buds (Table 1). In addition, congenital defects such as omphalocele also occurred while numerous cases of non-specific defects were observed. The induction of neural tube defects in the chicken embryos was also concentration dependent (Fig. 1; $p = 0.08$, Fisher's exact probability test). HNV was not only teratogenic, but also inhibited normal growth in the developing embryos in a dose-dependent manner (Fig. 2). The length, toe length, beak length and mass of the embryos were all affected by HNV. Although there was a strong tendency for HNV to inhibit growth in general, none of the observed effects were significantly different.

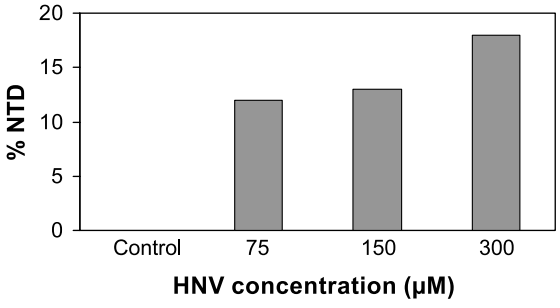

Fig. 1. The incidence of neural tube defects in chicken embryos treated with different concentrations of 3-hydroxynorvaline. The HNV concentration is given as the approximate final concentration after the HNV was absorbed into the egg. Control (n) = 14; 75 μM (n) = 16; 150 μM (n) = 17; 300 μM (n) = 17. ($p = 0.08$, Fisher's exact probability test)

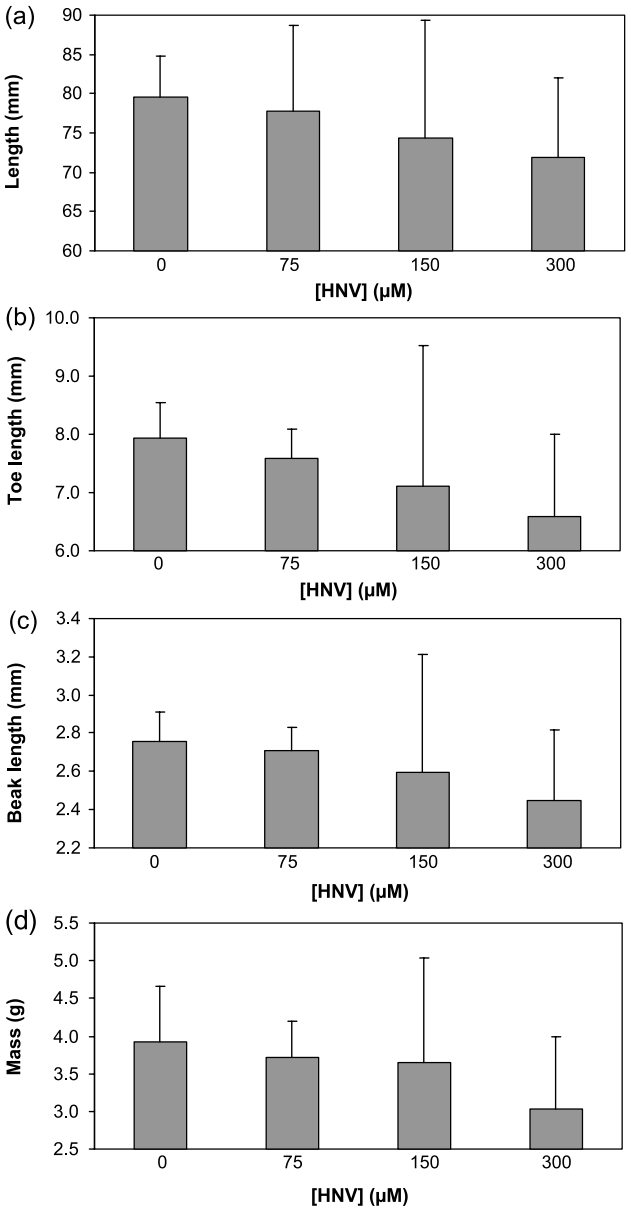

Fig. 2. The effect of 3-hydroxynorvaline on different parameters of the 10-day old chicken embryo: (**a**) body length; (**b**) toe length; (**c**) beak length; (**d**) body mass. The HNV concentrations are given as estimated final concentrations after the HNV has dissolved in the total volume of the egg. Control (n) = 14; 75 μM (n) = 16; 150 μM (n) = 17; 300 μM (n) = 17. Results are given as the mean ± standard error of the mean

HNV induced neural tube defects in developing mouse embryos in a concentration dependent manner (Fig. 3; $p < 0.001$, Fisher's exact probability test). Up to 17%

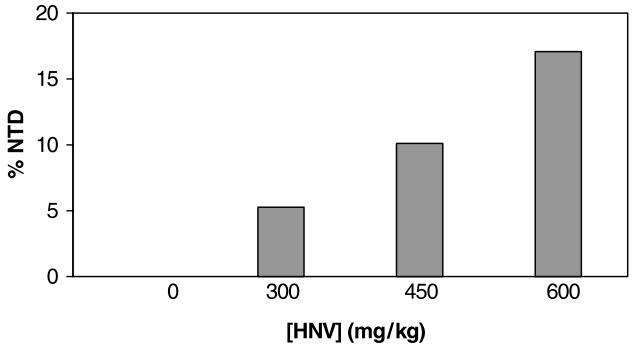

Fig. 3. The incidence of neural tube defects in mouse embryos treated with increasing 3-hydroxynorvaline concentrations. Control (n) = 112; 300 mg/kg (n) = 38; 450 mg/kg (n) = 99; 600 mg/kg (n) = 41. ($p < 0.001$, Fisher's exact probability test)

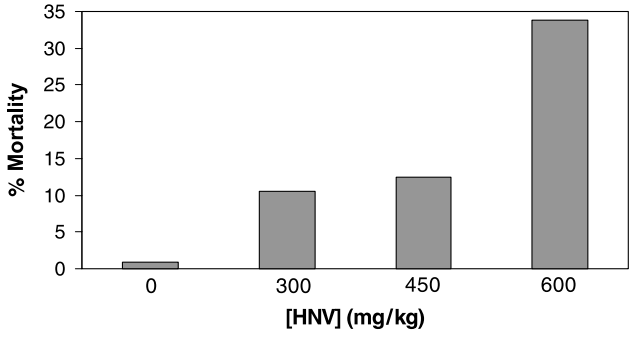

Fig. 4. Concentration dependent toxicity of 3-hydroxynorvaline in the mouse embryo model. Control (n) = 113; 300 mg/kg (n) = 38; 450 mg/kg (n) = 113; 600 mg/kg (n) = 62. ($p < 0.001$, Fisher's exact probability test)

neural tube defects was induced in the highest concentration group (600 mg/kg HNV), while no defects were observed in the control group. HNV also had a statistically significant toxic effect on the mouse embryos ($p < 0.001$, Fisher's exact probability test) and up to 34% of the embryos in the highest concentration group (600 mg/kg) died due to toxic effects of HNV (Fig. 4). Embryonic growth, as measured by the mean body mass of the embryos, was also inhibited in a concentration dependent manner (Fig. 5; $p < 0.001$, Kruskal-Wallis ANOVA).

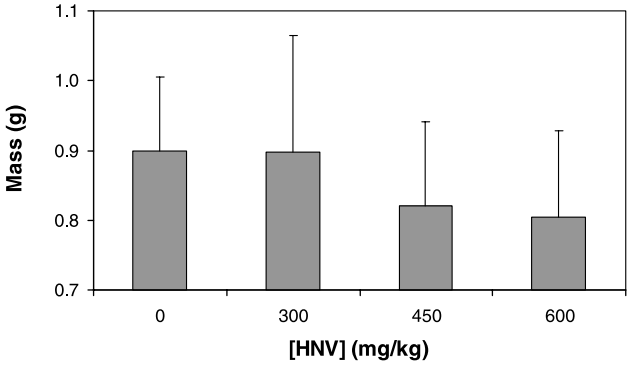

Fig. 5. Concentration dependent effect of HNV on the mean mass of developing mouse embryos. Control (n) = 76; 300 mg/kg (n) = 35; 450 mg/kg (n) = 48; 600 mg/kg (n) = 25. Values are given as the mean ± standard error of the mean. ($p < 0.001$, Kruskal Wallis non-parametric test)

Discussion

Chicken and mouse embryo models are used extensively to investigate the aetiology and mechanisms of neural tube defects. Since neurulation in these models are very similar to that in humans, these models are generally regarded as the most suitable for studying neural tube defects (George and McLone, 1995). The chicken embryo model is useful in applied teratological studies, since the embryos are sensitive to a variety of drugs (Gebhardt, 1972).

HNV was clearly teratogenic in chicken embryos and caused a relatively high incidence of neural tube defects. The effects were concentration dependent; the highest concentration caused neural tube defects in 18.5% of the embryos. HNV also impeded embryo growth in a dose-dependent manner. Measurements of body mass, body length, beak length and toe length were all inhibited.

A big disadvantage of the *in ovo* chicken embryo model that we experienced was the large variations in measurements of consecutive experiments. This may be the result of all the embryos not being exposed to the same concentrations of HNV. There can be several reasons for that. During dosage of the eggs, 50 μl of the HNV solution was injected into the air sack and came to rest on the inner membrane at the bottom of the air sac. The position of the embryo on this membrane, relative to the toxin droplet, may differ considerably from egg to egg. In order for HNV to come into contact with the embryo and be absorbed, it must diffuse through the membrane into the egg contents (i.e. egg yolk, etc). Embryos are embeded in different positions on the membrane. Depending on the distance form the droplet, embryos will be exposed to more or less of the HNV. The intra- and interbatch size of the eggs may also vary considerably. This in itself may have resulted in differences in concentrations within the eggs. Even distribution of HNV in smaller eggs will result in a higher final concentration. Supporting these assumptions is the fact that the standard deviations in the control groups were smaller than that of the groups receiving HNV, especially in the 150 and 300 μM groups. For example, some of the embryos in the treatment groups weighed the same as the controls, while others weighed markedly less.

Potential intra-individual differences in the genetic predisposition of embryos to the toxic effects of HNV may also play a part in the variation in the measurements.

The difficulties that we encountered with the chicken embryo model forced us to rather use the mouse embryo model. This model closely resembles the effects of the exposure of the human embryo/fetus to variations in the physiological, biochemical and metabolic characteristics in the mother's uterus. Pregnant females were dosed with HNV per body mass. It is therefore not unreasonable to argue that the final HNV concentration in all the mice were similar and/or had a much smaller variation than the chicken embryo model. HNV was also teratogenic in mouse embryos. Treatment with HNV dose-dependently increased neural tube defects (Fig. 3), caused increased deaths in embryos (Fig. 4) and decreased body mass of embryos (Fig. 5). In general, the mouse embryo model was the better model in this type of investigation when compared to chicken embryos. The concentrations of HNV, and subsequently its metabolic derivatives to which individual embryos were exposed to at the various dosage levels, probably displayed smaller variations than what occurred in the chicken embryo model. In addition, the more pronounced dose-response effect in the mouse embryo model can be readily ascribed to the higher quality of teratological data generated with this model.

We conclude that HNV, an L-threonine analogue, is teratogenic in developing chicken- and mouse embryos. HNV not only induced neural tube defects, but congenital defects, embryo toxic effects, and non-specific defects as well. HNV did not appear to be a highly specific neural tube defect inducing agent, but also displayed toxicity to developing chicken and mouse embryos, causing high incidences of mortality.

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