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ORIGINAL ARTICLE

The effect of hyperglycinemic treatment in captive-bred Vervet monkeys (*Chlorocebus aethiops*)

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Abstract

Nonketotic hyperglycinemia (NKH) is a neuro-metabolic disorder caused by a deficiency in the glycine cleavage system (GCS) and glycine transporter 1 (GlyT1). A case of atypical late onset of NKH has been reported in a colony of captive-bred Vervet monkeys. The purpose of this study was to evaluate the effect of sodium benzoate and dextromethorphan in reducing glycine levels in hyperglycinemic monkeys. Twelve captive-bred Vervet monkeys were assigned into three groups consisting of four animals (control, valproate induced and cataract with spontaneous hyperglycinemia). Valproate was used to elevate glycine levels and the induced group was then treated with sodium benzoate and dextromethorphan together with group three to normalise glycine levels in cerebrospinal fluid (CSF) and plasma. Valproate induction elicited changes in phosphate, alkaline phosphatase and platelet count, however, no significant changes in the glycine levels were observed, and this might be due to the individual variability within the group. The treatment intervention was only obtained in the spontaneous group whereby the glycine levels were normalised in CSF and plasma. Therefore, it can be concluded that sodium benzoate and dextromethorphan treatment was effective and beneficial to the hyperglycinemic group.

Keywords Dextromethorphan · Glycine · Sodium benzoate · Spontaneous hyperglycinemia · Valproate and Vervet monkey

Introduction

Nonketotic hyperglycinemia (NKH) is a rare autosomal recessive disorder characterised by high glycine levels in plasma and cerebrospinal fluid (CSF) (Applegarth and Toone 2006; Hennermann et al. 2012). There are two different forms of NKH; classical (neonatal) and atypical form (infantile, late onset and transient) (Kure et al. 2006). Clinical presentation of NKH differs for each form (Roy et al. 2003) and is categorized according to age of onset, clinical presentation and the severity of the disease (Bhamkar and Colaco 2007; Ramirez et al. 2012). Patients with classical form of NKH show severe neurological symptoms such as apnea, hypotonia, lethargy, myoclonic jerks, seizures and respiratory distress in the first few hours to days (Hoover-Fong et al. 2004; Hennermann et al. 2012; Roy et al. 2003). The late onset of atypical NKH is presented in children from two years with cognitive decline and behavioural problems (Hennermann 2006), however, patients tend to have a normal quality lifespan without the occurrence of seizures (Dinopoulos et al. 2005).

There is currently no cure for NKH, however, a combination of sodium benzoate and dextromethorphan is used to normalise glycine levels (CSF: plasma ratio < 0.02) (Arnold et al. 1997; Hussain et al. 2013; Hoover-Fong et al. 2004). Sodium benzoate is activated to benzyl-CoA in the liver through conjugation with glycine to form hippurate (Gregus et al. 1998). This mechanism eliminates glycine thereby reducing glycine levels in blood, urine and CSF (Neuberger et al. 2000; Van Hove et al. 2005). Dextromethorphan is used as an antagonist for N-methyl-D-aspartate (NMDA) and to minimize seizures in NKH patients (Randak et al. 2000; Hamosh et al. 1992). Although this treatment has been reported to have a neurological improvement effect on classical NKH new-borns, there are still conflicting findings about the effectiveness in severe cases of NKH (Van Hove et al. 2005; Randak et al. 2000; Neuberger et al. 2000). On the contrary,

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Hamosh et al. (1998) reported an improved arousal, decreased or eliminated seizures, and some developmental progress in four treated NKH infants. Based on Hamosh's findings, it is evident that the treatment is effective in some cases.

A case of late-onset atypical NKH has been reported at the Primate Unit and Delft Animal Centre (PUDAC) of the *South African* Medical Research Council (*SA*MRC) (Chauke et al. 2016). This form of NKH was observed when the animals were above the age of 5 years which is equivalent to an adult age in humans. The same hyperglycinemic monkeys also suffered from congenital cataract, which is a pathological condition that results in clouding of the normally clear crystalline lens of the eye (Hejtmancik 2008; Churchill and Graw 2011). The onset of the total cataract phenotype was detected microscopically at an age between three months to one year, however, their parents were asymptomatic (De Villiers et al. 2001; Magwebu et al. 2018). Currently, there are no studies that directly link hyperglycinemia to cataract formation, though a correlation has previously been suggested (Chauke et al. 2016). The captive-bred Vervet monkeys maintained in this facility are mainly used for biomedical research, therefore, any metabolic diseases may not be favourable for certain research studies hence an intervention approach was necessary to manage hyperglycinemia in this Vervet colony. Thus, the purpose of this study was to determine the effectiveness of sodium benzoate and dextromethorphan in reducing glycine levels in hyperglycinemic and induced Vervet model.

Materials and methods

Animal ethics

The Vervet monkeys are housed and maintained according to the South African National Standard for the Care and Use of Animals for Scientific Purposes (The SANS 10386:2008). The ethical procedure (Ref.08/13) and housing conditions (temperature, humidity, air changes/h and a photoperiod) were similar to the one reported by Chauke et al. (2016). The selected monkeys received a maintenance diet of pre-packed monkey chow maize meal (Avi-Products, South Africa) supplemented with vitamin C and D3. An additional fruit portion was offered mid-afternoon and a mixture of ground and whole kernel maize was later provided as an enrichment to allow foraging. All diets were prepared at PUDAC and water was available ad lib via an automatic watering device.

Formulations and administration of compounds

Valproate (50 mg/kg/day) and sodium benzoate (250 mg/kg/day) (Sigma Aldrich, South Africa) were supplied in a powder form and dextromethorphan (5 mg/kg/day) (Johnson & Johnson, South Africa) was provided as a syrup. An exact amount of compound powder or syrup based on each animal's body weight was weighed and mixed to a 30 g portion of food. The compounds were administered orally in food of the treated subjects once daily in the mornings.

Selection criteria

Twelve adult Vervet monkeys were selected based on their glycine levels and cataract phenotype. The animals were assigned into group 1 (control), group 2 (induced) and group 3 (cataract with spontaneous hyperglycinemia) and each group consisted of four individuals. The control and induced groups were healthy individuals without cataract and glycine levels in CSF were normal, whereas the spontaneous group was presented with both total cataract phenotype and hyperglycinemia. Due to the shortage of the animals in the same age group (10–15 years) as the hyperglycinemic individuals, the majority of the selected animals were females especially for the induced group. An animal intervention approach with valproate, sodium benzoate and dextromethorphan was followed. The control group received a maintenance diet throughout the study and group 2 was supplemented with valproate (50 mg/kg/day) for three weeks. Sodium benzoate (250 mg/kg/day) and dextromethorphan (5 mg/kg/day) were administered for four weeks as treatment for both the induced and spontaneous group followed by the washout period of four weeks. The effective dosages were chosen for each compound based on previous studies (Arnold et al. 1997; Van Hove et al. 2005; Viljoen et al. 2012).

Sample collection

Blood collection

Blood (2–4 ml) was obtained via femoral venipuncture after Ketamine anaesthesia at 10 mg/kg (Kyron laboratories, South Africa) (Chauke et al. 2016). On each day of sampling, monkeys received the total dose of the compound in small food balls and blood was taken two hours after compound administration. Blood for plasma isolation was collected in EDTA-containing tubes. Plasma was isolated at 4 °C (1400 x g, 10 min) and maintained at −80 °C for glycine analysis. Blood sampling (2.2 ml) for gene expression was collected in PAXgene Blood RNA Tubes (BRT) (PreAnalytiX, Switzerland) and stored at room temperature for two hours prior being stored at −80 °C. Blood (2 ml) was also collected into SST and EDTA tubes to perform clinical biochemistry and haematology tests. The clinical chemistry included test for bilirubin (total and direct), alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatise (ALP), total protein, albumin, globulin, cholesterol (total, LDL-C, HDL-C), urea, creatinine, triglyceride, chloride, potassium, calcium and sodium. In addition, complete

blood count examination for haematology tests included red blood cells (RBC), white blood cells (WBC), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH); mean corpuscular haemoglobin concentration (MCHC), heamatocrit (HCT), red blood cell distribution (RDW), haemoglobin (Hb), neutrophils, eosinophils, basophils, lymphocytes, monocytes and platelets count.

Cerebrospinal fluid (CSF) collection

Cerebrospinal fluid (CSF) was collected at baseline, at the end of drug intervention and after washout period by inserting a needle in the cisterna magna to aspirate the sample which was then transferred into a sterile 1.5 ml cryotubes and stored on ice for glycine analysis. The syringe was removed, and pressure was applied to the area of puncture.

Urine collection

Urine (24 h) samples were collected after the morning feed by placing a sterile collection pans underneath the cages with a gritted funnel to prevent food and faeces contamination. Urine was then directly collected via the funnel into a sterile bottle.

Clinical observations

At the time of blood sampling (baseline-washout), the weight of each animal, body temperature, heart and respiration rate and blood pressure were recorded. Additionally, the general well-being and food intake for all the groups were monitored and recorded daily.

Statistical analysis

Data was presented as means ± SD. The MedCalc® programme version 10.4.0.0 (MedCalc Software,Mariakerke, Belgium) was used for basic statistical analyses. Any significant differences ($P < 0.05$) for ANOVA analysis between groups were tested by two-tailed t-test using GraphPad Prism version 6.00 for Windows (La Jolla California USA).

Results

The biochemical analysis showed a significant difference for urea ($P = 0.02$) at baseline between the groups (Fig. 1). The remaining biochemical and haematological parameters were not significant; however, valproate specific changes were noted during the induction period for ALP, phosphate and platelet count. The levels of ALP were elevated during the induction and treatment phase (Fig. 2). The phosphate levels (Fig. 3) increased by 54% from baseline to induction period and the treatment reduced phosphate levels by 31%. Similarly, platelet

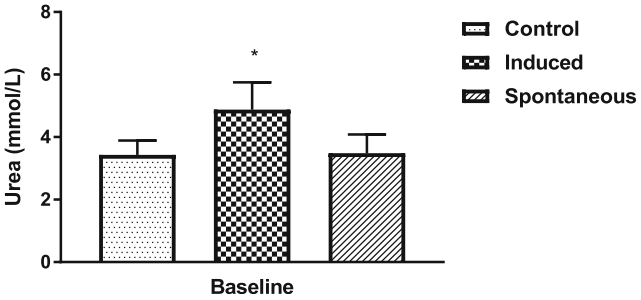

Fig. 1 Urea. Baseline results for control, induced and spontaneous groups: the control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50 mg/kg/day for three weeks followed by treatment with sodium benzoate which was also given to the spontaneous group at 250 mg/kg/day and dextromethorphan (5 mg/kg/day) for four weeks. * represent significant difference ($p < 0.05$)

count (Fig. 4) indicated a 30% increase from baseline to induction phase and was reduced by 12% after treatment. Nevertheless, valproate induction did not elevate glycine levels in plasma nor CSF. However, there was a significant difference in CSF ($P < 0.001$) between the groups. The significant changes were observed in the spontaneous group, which had high baseline glycine levels in CSF (>10 μmol/L) compared to the control group (8.55 μmol/L) (Fig. 5). Additionally, a significant difference was observed from baseline to treatment for plasma ($p < 0.007$) (Fig. 6a) and CSF ($p < 0.04$) (Fig. 6b) in the spontaneous group. The urine analysis for glycine was inconclusive and excluded from the analysis.

Discussion

Cataract has been an ongoing disorder since the early stages of life in all the affected monkeys maintained at PUDAC (de Villiers et al. 2001), however, hyperglycinemia was observed at a later stage (>5 years) with no clinical signs such as lethargy, hypotonia, seizures and apnea which are common in NKH neonates (Boneh et al. 2005). Although some of the

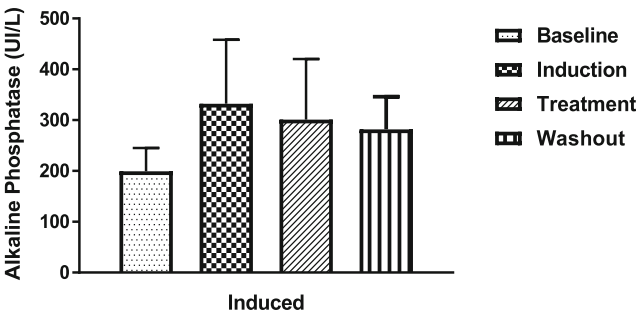

Fig. 2 Alkaline phosphatase (ALP). The induced group was given valproate at 50 mg/kg/day for three weeks and treated with sodium benzoate at 250 mg/kg/day and 5 mg/kg/day dextromethorphan for four weeks. Washout period was four weeks

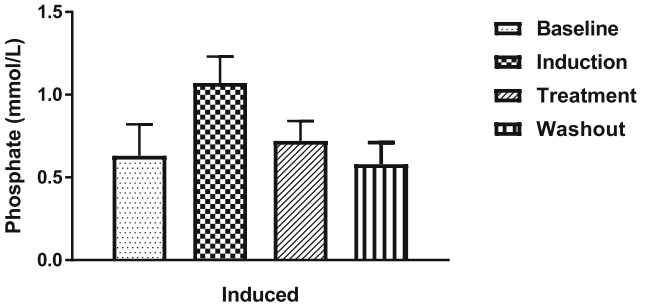

Fig. 3 Phosphate. The induced group was given valproate at 50 mg/kg/day for three weeks and treated with sodium benzoate at 250 mg/kg/day and 5 mg/kg/day dextromethorphan for four weeks. Washout period was four weeks

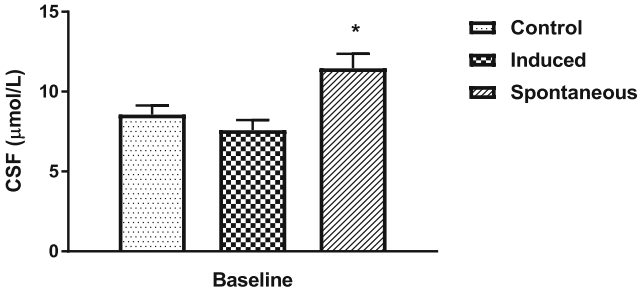

Fig. 5 Glycine in CSF of Vervet monkeys. Baseline results for control, induced and spontaneous groups: the control group received a maintenance diet throughout the intervention study. The induced group received valproate at 50 mg/kg/day for three weeks followed by a treatment with sodium benzoate which was also given to the spontaneous group at 250 mg/kg/day and dextromethorphan (5 mg/kg/day) for four weeks. * represent significant difference ($p < 0.05$)

cataract siblings died immediately after birth before they could be screened for mutations and glycine levels, jerky movements were observed in some of the surviving siblings (Chauke et al. 2016). Human patients with the late form of NKH tend to have a normal life without presenting symptoms (Dinopoulos et al. 2005), and this could be the case with the hyperglycinemic Vervet monkeys.

The first initiative of this study was to induce hyperglycinemia in healthy Vervet monkeys using valproate and normalise glycine levels of the spontaneous hyperglycinemic group using the available NKH treatment. Valproate, which is responsible for serious adverse reactions such as hyperammonemia, hepatotoxicity and thrombocytopenia (Star et al. 2014; Koenig et al. 2006) was used to mimic the spontaneous hyperglycinemic model. Since valproate is also known to have an impact on the kidney and liver biomarkers, a small dose (50 mg/kg) was chosen to induce hyperglycinemia over a short period of time.

As mentioned, the reported biochemical and haematological changes were mainly due to valproate induction (50 mg/kg), however, adverse reactions such as hepatotoxicity were not observed in the induced group. Although there were no significant clinical signs of liver damage in this study due to the small dose and short intervention period, valproate showed potential in damaging the liver with ALP levels slightly elevated in the induced group (Fig. 2). Since ALP originates from the liver and bone, an increase in ALP levels without elevated GGT levels means that valproate might have an impact in the liver (Ahmed and Siddiqi 2006). Additionally, valproate slightly elevated platelet counts, and this response has not been reported in association with valproate therapy in humans. Most studies have indicated that valproate causes thrombocytopenia which normally occurs several months after valproate therapy (Espandiari et al. 2008; Hauser et al. 1996; Nasreddine and Beydoun 2008). The degree of thrombocytopenia correlates with the levels of valproate and is very common in pediatric patients with an incident of 5–40% (Delgado et al. 1994; Karaoglu et al. 2009). Nevertheless, PUDAC in-house Vervet reference values for haematology

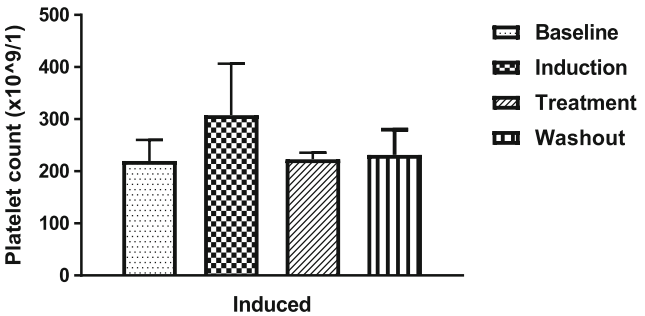

Fig. 4 Platelet count. The induced group was given valproate at 50 mg/kg/day for three weeks and treated with sodium benzoate at 250 mg/kg/day and 5 mg/kg/day dextromethorphan for four weeks. Washout period was four weeks

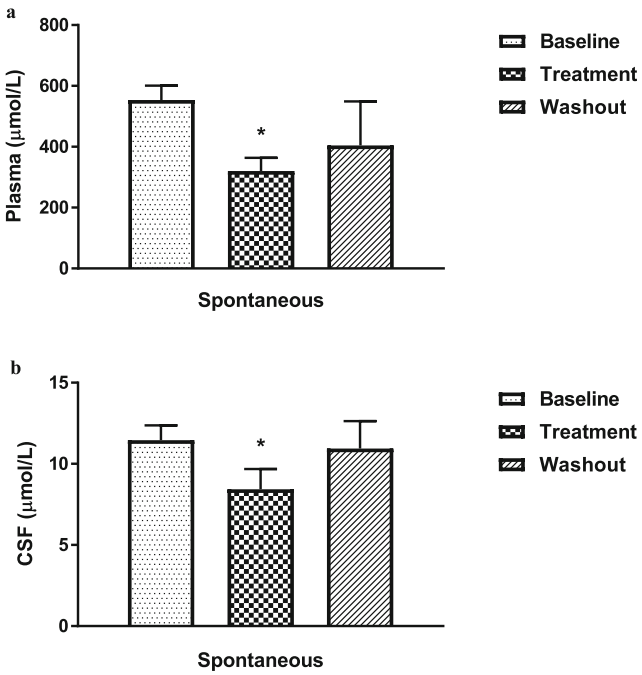

Fig. 6 Glycine analysis for the spontaneous group. (a) Plasma and (b) CSF. The spontaneous group was treated with sodium benzoate at 250 mg/kg/day and dextromethorphan (5 mg/kg) for four weeks. * represent significant difference ($p < 0.05$)

and biochemical parameters were used to determine whether the observed changes in this study were detrimental. Although phosphate (0.63 ± 0.19 mmol/L) (Fig. 3) and platelet count levels (199.25 ± 46.00 × 10^9/1) (Fig. 4) were not significantly elevated during valproate administration and still fell within the normal levels of the in-house reference values (1.30 ± 0.29 mmol/L, 317.85 ± 50.89 × 10^9/1) respectively, these changes were reversed by the treatment.

With regard to glycine analysis, no significant changes were observed for valproate induced group, and this might be due to the individual variabilities (age, sex and genetics) within this induced group. Since the animals were from the same age group, our findings suggested that the variations might be of genetic origin. These findings were also supported by a study conducted in adult Iranian patients, whereby age or sex did not affect valproate pharmacodynamics (Aghebati et al. 2012). Since inter-individual variations are known to result in insufficient treatment outcomes in patients on valproate therapy (Chatzistefanidis et al. 2012), genetic screening for polymorphisms in genes known to be responsible for valproate glucuronidation; the UDP-glucuronosyltransferase family 1A (UGT1A6 and UGT1A9) was therefore necessary. To further investigate the effect of polymorphisms using these valproate glucuronidation genes, a genetic study was conducted using the same valproate-induced animals. In that study, two known polymorphisms (S7A and T181A) were identified in *UGT1A6* (unpublished data). Since these two polymorphisms have been reported to lower valproate serum levels (Guo et al. 2012; Hung et al. 2011), it can be postulated that S7A and T181A might have altered valproate metabolism, hence glycine levels in plasma and CSF were not significantly elevated in the induced group.

Furthermore, sodium benzoate and dextromethorphan normalised glycine levels (<350 μmol/L) in plasma and CSF (<10 μmol/L). However, this combination was only effective for the spontaneous compared to the induced group. A similar observation has been reported in human studies where CSF levels were not normalised by the NKH treatment (Hamosh et al. 1998; Kojima-Ishii et al. 2008; Madu and Oliver 2013). Based on these findings, sodium benzoate (250 mg/kg) and dextromethorphan (5 mg/kg) were found to be more effective in reducing glycine levels in the spontaneous group. The findings also showed that the effect of the NKH treatment is reversible in the Vervet model. Thus, sodium benzoate and dextromethorphan treatment must be used continuously in order to manage glycine levels in this Vervet colony.

Conclusion

In this study, administration of valproate, sodium benzoate and dextromethorphan did not show any signs of unwellbeing of the animals. The findings demonstrated that low dose (50 mg/kg) of valproate did not have any significant clinical impact on biochemical and hematological parameters even though changes were observed for ALP, phosphate and platelet count. However, these findings do not bring into disregard the efficacy of valproate but further studies using a higher dose, larger group size with both genders and extended induction period are suggested. Therefore, it can be concluded that the recommended dosage of sodium benzoate (250 mg/kg) and dextromethorphan (5 mg/kg) treatment is effective in normalizing glycine levels in hyperglycinemic Vervet monkeys.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was approved by the SAMRC Ethics Committee for Research on Animals (ECRA) (Ref.08/13). All procedure performed in study involving animals were in accordance with the ethical standards of the South African National Standard for the Care and Use of Animals for Scientific Purposes (South African Bureau of Standards, SANS 10386, 2008).

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