Bachelor of Science in Medicine Degree Program
End of Term Final Report

Student Name: Aidan Baker **Date:** 06/08/17

Project Title: Nimodipine therapy for juvenile ferrets with hydrocephalus

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Summary (250 words max single spaced):

Hydrocephalus causes damage to periventricular white matter via compression, stretching, waste material accumulation, and ischemia. Nimodipine has been shown to provide benefits clinically in subarachnoid hemorrhage and in young rats with hydrocephalus, presumably by causing vasodilatation and improving blood flow. Our hypothesis was that nimodipine would provide neuroprotection in ferret kits with induced hydrocephalus. Hydrocephalus was induced by kaolin injection into the cisterna magna of postnatal day (P14) ferrets. The drug trial started on P29 after animals were stratified using ventricle size based on magnetic resonance imaging (MRI). Kits were assigned to vehicle, low dose (3.2mg/kg/day), or high dose (16 mg/kg/day) treatment groups, which were administered in a blinded manner twice daily by subcutaneous injection. The drug trial was briefly discontinued due to toxicity caused by the solvent vehicle: dimethyl sulfoxide (DMSO). After the amount of DMSO was reduced the trial was restarted and ran for 14 days to P52. During the experiment behavioural analysis was done using two separate open field tests, every three days. Vision testing started once kits opened their eyes. There was no consistent or statistically significant difference between any of the treatment groups on either the open field tests or the visual test. We conclude that nimodipine administered in this manner does not seem to provide a neuroprotective effect in a ferret model of hydrocephalus. We cannot exclude the possibility that continuous or more frequent delivery, which would not easily be done in the animal model, might be efficacious.

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Introduction:

Hydrocephalus is a neurological disease characterized by abnormal flow of cerebral spinal fluid (CSF) in the brain. In infants and children it can present in the context of congenital malformations, following brain hemorrhage after premature birth, following meningitis, or in association with brain tumors. The abnormal flow leads to a build up of CSF in the ventricles causing damage. The damage that does occur is multifactorial; the axons in periventricular space are stretched and compressed, waste products accumulate in the CSF, and ischemic changes cause decreased white matter blood flow contributing to axonal and oligodendroglia damage.[1] Elevated calcium corresponds with an increase in the content of calpain in the white matter. Also noted was heightened immunoreactivity in periventricular axons in young hydrocephalic rats with notable axonal damage, suggesting that calcium mediated proteolysis is potentially associated with axonal cytoskeletal damage in hydrocephalus.[2] Damage seen in the animal model is similar to the damage seen in humans. Human studies showed that more severe hydrocephalus led to more symptomatic individuals, with symptomatic individuals having narrower corpus callosum, alveus and fornix. In both rats and humans it seems that decreased blood flow and increased calcium play a role in the destruction of the periventricular white matter.[3] The definitive treatment for hydrocephalus is neurosurgery, for the placement of a shunt. However, surgery is associated with complications for young infants especially those born preterm. Delaying surgery increases the likelihood of severe complications. Therefore being able to prevent damage from hydrocephalus to safely delay surgery would provide better outcomes for infants.[4]

Previously, young hydrocephalic rats were treated using magnesium sulfate ($MgSO_4$), a calcium channel blocker, chosen for its vasodilatory and calcium channel blocking properties. Rats treated with magnesium performed better on the rotating cylinder and water maze tests, with the corpus callosum being thicker in the treated cohort. When a high dose magnesium trial was done, the rats become lethargic due to the treatment and in turn performed worse in behavioural testing.[5] Due to the success in rats a trial was done in ferrets, which have gyrencephalic brain anatomy with white matter content more comparable to humans.[6] The treatment did not provide any significant benefit histologically or in terms of ventricle size. Additionally, treated ferrets were more lethargic, weighed less, and had reduced activity. Lethargy caused by the magnesium made it difficult for behavioural assessment to be done, the magnesium also caused dryness around the injection site.[7]

Nimodipine, a dihydropyridine L-type calcium channel blocker, has also been shown to be efficacious in the treatment of human neurological issues and has known cerebral vasodilation effects.[8,9] Nimodipine already has clinical significance in the setting of subarachnoid hemorrhage, by reducing vasospasm it was shown to improve clinical outcomes including: increasing a patients' likelihood to go home, have shorter ICU stays, and have better outcomes on the Glasgow Outcome Scale (GOS).[10] In addition, nimodipine has been evaluated in the settings of dementia and to improve regeneration after skull base fractures.[11]

Nimodipine was previously shown to be protective in young rats with kaolin induced hydrocephalus. [5] The treatment group had better behavioural outcomes, exhibiting significantly better performance staying on a rolling cylinder and were 70% faster in finding a hidden platform (Morris water maze test). At the end of the study, treated rats exhibited less spontaneous activity in an open field, possibly indicating that they were familiarised to the test environment. Untreated rats did not exhibit improved performance. Though there was no difference in

ventricle size or histology, the corpus callosum was thicker in rats treated with nimodipine, offering a potential explanation for the behavioural improvement.

Ferrets are born in a very immature state; therefore, they are useful animals to examine conditions that affect human fetuses and premature infants. At birth, the cellular differentiation and morphological features in the ferret brain are similar to those of second trimester human fetuses.[12] Many of the important events such as synaptogenesis, gliogenesis, and oligodendrocyte maturation occur in the postnatal period for ferrets, allowing examination of issues that occur in the human third trimester.[6] A model for induced hydrocephalus in ferrets has been previously described, using kaolin injected into the cisterna magna of P14 ferrets. Hydrocephalic ferrets displayed hyperactivity and lack of purposeful movement, moving more and spending less time trying to escape in open field experiments. They also displayed reduced weight gain.[13] In human infants, hydrocephalus causes difficulty in focusing and shifting attention, has an effect on social skills, gross motor skills, and to a lesser extent fine motor skills and language development.[10]

Based on the previous successful use of nimodipine in a rat model of hydrocephalus and its use as a neuroprotective agent in humans, we hypothesize that it will provide benefit to juvenile ferrets with induced hydrocephalus.[8] In addition we expect it to not have the same negative side effect as previously seen with $MgSO_4$ given to juvenile ferrets.[7]

Methods:

Animals:

Thirty-three pigmented sable ferret kits were obtained from Marshall Farms (North Rose, NY) at postnatal day 5-7 (P5-7). The kits stayed in cages with their mothers until P52, the cages were in a room on a 12-12H light dark cycle with at temperatures of 21-22˚C and 35-45% humidity. Food was provided ad libitum. For identification, the paws of the ferrets were tattooed. All animals were treated humanely according to the guidelines set forth by the Canadian Council on Animal Care. The University of Manitoba institutional animal ethics committee approved the experimental protocol (15-076). All precautions were taken to limit the number of animals and their suffering.

Hydrocephalus Induction:

Hydrocephalus was induced by injecting kaolin (aluminum silicate; Sigma, St. Louis MO), following the protocol previously described.[13] Kaolin causes fibrosis and inflammation in the CSF outflow track around the aperture of the 4th ventricle and basal subarachnoid space, causing an obstructive hydrocephalus.[14,15] Kaolin injections were performed on the kits at P14. They were anesthetized using 2.5% isoflurane in oxygen. The dorsum of their necks was shaved and cleaned using chlorhexidine. With their necks in a flexed position, a 27-gauge needle was used to inject 0.2ml of sterilized 20% kaolin in saline into the cisterna magna using aseptic technique. Controls were given sham injections of saline in the same format. During recovery, animals were given buprenorphine to ease the pain and observed in the post anesthesia period. Once recovered from the anesthesia they were returned to their mothers and monitored every 12 h for the next two days. During this time buprenorphine was used to control pain and 0.45% saline was used to prevent dehydration. Over the course of the experiment the animals were weighed daily and monitored. Those experiencing severe neurological

impairment, inability to groom or feed, or weight loss greater than 10% or on 3 consecutive days, were humanly sacrificed to prevent further suffering.

Magnetic resonance imaging:

To assess the severity of the hydrocephalus and to stratify the animals, MRI was used. Images were taken 2 days post injection (P16) to ensure that hydrocephalus had been induced. Images were again taken post injection at P29 to stratify the animals into treatment groups and a final MRI was taken on the day of sacrifice. MRI's were accomplished using T2-weighted coronal images of 1.0mm thickness using a 7 Tesla Bruker Biospec/3 MR scanner (Karlsruhe, Germany). During imaging, the ferrets were anesthetized using ~5% isoflurane in O_2/NO_2 and maintained using ~2.5% isoflurane in O_2/NO_2 delivered using a nose cone. Vitals of respiration rate and body temperature were monitored using a small animal and gating system (SA Instruments Inc., Stony Brook, NY). To maintain a 37°C temperature, a circulator warming pad was used (ThermoScientific HAAKE, Karlsruhe, Germany). The supine animals were placed in appropriately sized coils as their heads grew.

The size of the ventricles was measured using Marevisi software (NRC, Winnipeg, MB, Canada). The relative size of the lateral ventricles was calculated by measuring the area of the frontal horns immediately caudal to the optic chiasm. This value was then divided by the cerebrum area. The area ratio has previously been shown to correlate extremely well with the ventricle volume.

Drug Treatment:

The nimodipine (Sigma, St. Louis, MO) and vehicle treatments were administered beginning 17 days after kaolin injection at P29-31 (n=9 for sham (B), n=9 low dose (A), and n=8 high dose (C)). Ferrets were weighed daily and these weights were used to calculate the volume of the treatment. Solutions of nimodipine and vehicle were prepared in 50% dimethyl sulfoxide (DMSO; Fisher Scientific, Nepean ON) and 10% ethanol in 0.9% NaCl / distilled water at room temperature and subsequently stored at 4°C. The solutions were labeled A-C and were administered blindly at 1mL/100mg twice a day via subcutaneous injection. The nimodipine doses were 3.2mg/kg/day or 16mg/kg/day for the low and high dose respectively. The doses were based on previous rodent studies, which showed 20mg/kg/day to be protective. The drug treatment was interrupted by adverse effects, particularly lethargy. This led to 3 ferrets dying and 6 more reaching humane end points. The drug vehicle was reformulated so that lower volumes could be used. After 9 days, the drug trial was continued at the same nimodipine dose for the last 14 days, with the remaining ferrets (n=17). Injections were given in the morning before behavioural testing or MR imaging and then again in the afternoon/evening.

Behavioural testing:

Previous studies with hydrocephalic ferrets showed that they differ from controls in a few behavioural tests.[16] Testing was started at P9-11 and was done every 3 days until P42-44, all tests were done on the same day. Two apparatuses were used to determine open field ambulatory behaviour. The first was an enclosed box (44cm x 43cm x 29cm) with 15 light beam sensors on each axis to quantify vertical, ambulatory, and total movements (Opto-Varimex 3; Columbus Instruments International Corp., Columbus, OH, USA) during a 3-minute period. The

second apparatus was a chamber (75cm x 75cm x 45cm) in which the kits were videotaped for 3 minutes to track the motion using a HVS Image 2100 Plus Tracking System software (HVS Image Ltd, Twickenham, Middlesex, UK). Motion was quantified by dividing the box into 100 squares of 7.5 x 7.5cm and recording how many squares they entered, the total distance they moved, and percentage of squares entered. Qualitative evaluations of pivoting, crawling, walking, and rearing were also performed.

Vision was tested when the ferrets started to open their eyes (P36). A previous experiment led to the suspicion that hydrocephalic kits were going blind, thus affecting the outcomes of the behavioural tests. Vision was tested using a Styrofoam ball (5 cm diameter, painted red) attached to the end of a metal stick. The ball was introduced in two different manners. First, it was held at the side of the ferret and waved until the animal turned its head toward the ball (Figure 1). Care was taken to avoid excessive noise or other sensory clues. Second, the ball was placed in front of the kit then moved to see if the ferrets would track the ball. Outcomes were reported semi-quantitatively with three categories: normal visual response (0), abnormal visual response (1), and no visual response (2).

Sacrifice:

Ferrets were euthanized within 24h of the final MRI at P52 or when humane endpoints were met. Euthanasia was accomplished by administering 5% isoflurane in oxygen followed by carbon dioxide narcosis and exsanguination by transcardiac perfusion using ice cold 0.1M phosphate buffered saline (PBS). The brains were removed for histologic and biochemical studies, which are not part of this project.

Statistics:

All data are presented as mean ± standard error of mean (SEM). Weight, behavioral, and ventricle size data were analyzed using ANOVA, with $p \leq 0.05$ considered significant. Vision data were analyzed using Chi-Squared analysis. Statistical analysis was done using Microsoft Excel.

Results:

Mortality:

Following induction of hydrocephalus in 35 kits, 9 died within a few days due to complications of the kaolin injection. After the drug trial was started at P29-31, 3 kits died and another 6 had to be euthanized because of excessive weight loss. After the volume of DMSO vehicle was reduced, no further deaths occurred until the end of the study when the kits were MR imaged and subsequently sacrificed.

Magnetic Resonance Imaging:

Two days after kaolin was injected there was a significant difference between the ventricle size of saline-injected (non-hydrocephalic) controls and hydrocephalic kits. The difference remained significant throughout the trial. Ventricle size of the three hydrocephalic treatment groups (sham, low dose and high dose nimodipine) was designed to be the same at P29. All three

hydrocephalic treatment groups displayed ventricular growth during the drug trial, but there was no difference between treatment groups at the end of the drug trial.

Weight:

There was no significant difference in weights between the control and hydrocephalic kits, although the hydrocephalic animals tended to weigh less, or between the treatment groups. Both control and hydrocephalic kits increased in weight over the course of the trial (Figure 3).

Behaviour:

As suspected from previous experiments, 4 of the hydrocephalic kits had gone blind and were unable to respond to the visual challenge. All 4 had severe hydrocephalus based on MR images taken at time of sacrifice. None of the non-hydrocephalic control animals or kits with moderate hydrocephalus suffered vision loss. There was no statistical significance between groups.

Kits started to crawl between P26-30 and started walking between P34-38, which is within the normal range previously reported.[16] There was no significant difference in developmental milestones between any of the treatment groups regardless of degree of hydrocephalus. While most of the kits progressed in their ability to walk, those with severe hydrocephalus began to show an unsteady gait.

Differences of the movement pattern of the control and hydrocephalus are shown in Figure 4. The control animals spent time examining the box and looking out of the open field box, moving in a purposeful manner to explore around the open field experiment. Hydrocephalic animals displayed similar patterns of behaviour to control animals unless they had gone blind. Blind ferrets (see last frame in figure 4) ran around the edge of the open field experiment bumping into the walls and spending no time looking out of the open field area. This had been interpreted as hyperactivity in a previous study.[13]

Statistical analysis on the data from the behavioural test showed that there was minimal difference between the control and hydrocephalic groups. On the last testing day, the control animals reared more than the hydrocephalic animals. When compared to one another the drug groups showed minor differences (Figure 5). There was some difference noted between the sham and high dose group on a few tests, with the high dose treatment group scoring higher on the ambulatory and total movement measures on the second to last testing day. No significant differences were noted in the open field experiment monitored by the HVS Image 2100 Plus Tracking System.

Discussion:

Effects of Hydrocephalus on Brain and Behavior

Hydrocephalus causes motor deficits by damaging intracortical and corticospinal pathways.[1] In terms of motor deficits there was very minor difference between the control and hydrocephalic kits, with both reaching developmental milestones at the same point and performing similar in the open field experiment. On the final day however, there was a difference in rearing activity between the two categories. Hydrocephalus also causes memory deficits by destroying the

fimbria-fornix which connects the hippocampus to sub-cortical areas. We did not test memory because there are no well-validated tests for very young ferrets. A previous study documented marked hyperactivity of hydrocephalic ferrets, however this was not noted in this study.[13] In the same study it was noticed that hydrocephalic ferrets spent less time trying to escape the open field area, indicated by such behaviours such as rearing and looking out of the open field area.[13] This pattern of behaviour was observed in this study only in ferrets that had gone blind.

Hydrocephalus can cause blindness through several mechanisms. Raised intracranial pressure can causing papilledema (retinal damage), posterior cerebral artery ischemia, or destruction of the intracortical optic radiations. Histologic examination of brains in the previous study, showed destruction of periventricular cortical white matter in the occipital lobe. Therefore this is cortical blindness.[13] Visual cortex damage with severe atrophy and disorganization is also described in juvenile cats with induced hydrocephalus.[17] Although use of the red ball is a relatively crude test, it seemed to be effective as we could detect kits that had lost visual acuity before they had gone blind. The test fell short in its ease to elicit a strong response, making it rather subjective at times as to whether the kit was disinterested (e.g. an effect of lethargy) or truly lacked vision. This was somewhat mitigated by incorporating the tracking component as kits seemed more inclined to track than orientate.

Nimodipine Treatment:

Nimodipine was previously shown to provide some benefit in treating juvenile rats with hydrocephalus.[3] As an L-type calcium channel blocker that increases cerebral blood flow, nimodipine was tested because reduced blood flow is thought to cause white matter damage in hydrocephalic brains.[2,9] It was anticipated that nimodipine would provide benefit to ferret kits with induced hydrocephalus. However, we found that drug treatment at low and high doses had no statistically significant effect on the ventricle size, body weight, or behavioural scores. The failure to show benefit could be on several grounds (see below).

Difficulties in Translation from Rodents to Humans:

The Stroke Therapy Academic Industry Roundtable in 1999 concluded that there is a need for a larger animal model in-between rodent experiments and human clinical trials.[18] because hundreds of drugs that seemed to benefit mice and rats with ischemic brain damage failed in human clinical trials.[19] There are many possible reasons for the failure seen in stroke pre-clinical experiments. Among these are the fact that animals used in drug trials are otherwise healthy rather than facing complex issues seen in human patients and of different relative age.[20] Considering the need for a proper intermediate species in hydrocephalus research, ferrets were chosen as they have been noted to be good for translational purposes as their neurodevelopment makes them favorable to studying second and third trimester fetal issues.[6] Additionally, MRI studies in ferrets translate favorably to the human context.[21] Previous work showed that the brain damage and behavioural complications seen in hydrocephalic ferret kits are similar to that seen in human infants[13], making ferrets a reasonable choice to bridge the gap between rat and human or further large animal studies.

Problems with the Nimodipine Trial / Study Limitations:

We designed the nimodipine dose and the DMSO solvent vehicle based upon a previously efficacious experiment in young rats. Unlike anything ever witnessed in rats, the DMSO caused severe morbidity and some mortality in the young ferrets in the first drug trial got very sick. The room in which the animals were housed had a noticeable sulphur like odor indicative of excreted DMSO. At this time, many of the animals became ill either dying or having to be euthanized due to weight loss. There are no data regarding the comparative toxicity of DMSO between rats and young ferrets; we cannot exclude the possibility that DMSO, a commonly used solvent, is particularly harmful to immature ferrets. After this point the amount of DMSO used to dissolve the nimodipine was lowered and there was no further acute toxicity. Unfortunately, this complication shortened the drug trial, originally planned for 3 weeks, to 2 weeks. It also reduced the sample size in each group, perhaps making it more difficult to detect a benefit from a statistical stand point. Another problem with the drug trial was the administration method. Nimodipine has a relatively short half life on the order of a few hours following intravenous injections.[22] In the drug trial, injections were given on a 12-hour schedule, likely associated with significant periods with less than ideal drug levels. Subcutaneous injections have been shown to improve peak concentrations and prolong the half-life of nimodipine in rabbits.[23] Osmotic pumps were considered as a possible method of drug delivery. However, none have the capacity to deliver sufficient volume over several weeks.

Conclusion:

Nimodipine administered by repeated subcutaneous injections was not shown to improve the behavioural outcomes or ventricle size of hydrocephalic ferret kits, despite promising results in a rat model of hydrocephalus. However, the results from this study do not provide conclusive evidence for lack of efficacy of nimodipine in treating hydrocephalus due to the limitations in the method of drug delivery.

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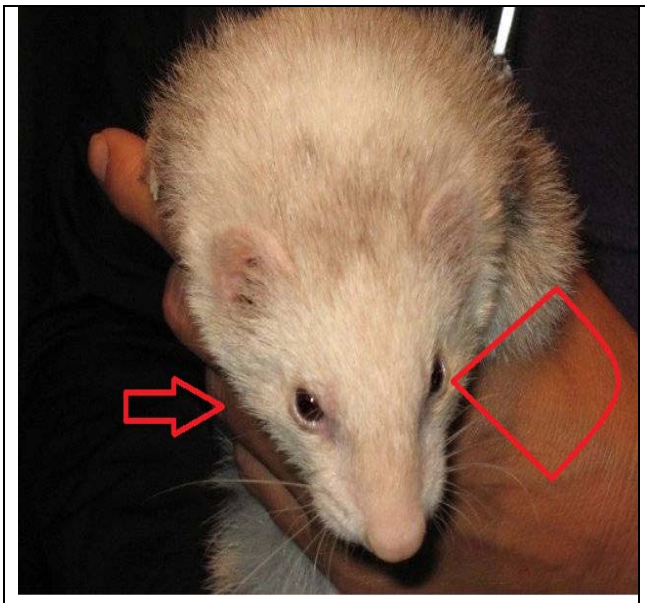

Figure 1: To test vision, a red ball was introduced towards the ferret kit in direction of the arrow at which point it was wiggled in the angle and arc approximated. Note: an image of an adult ferret was used for the figure.

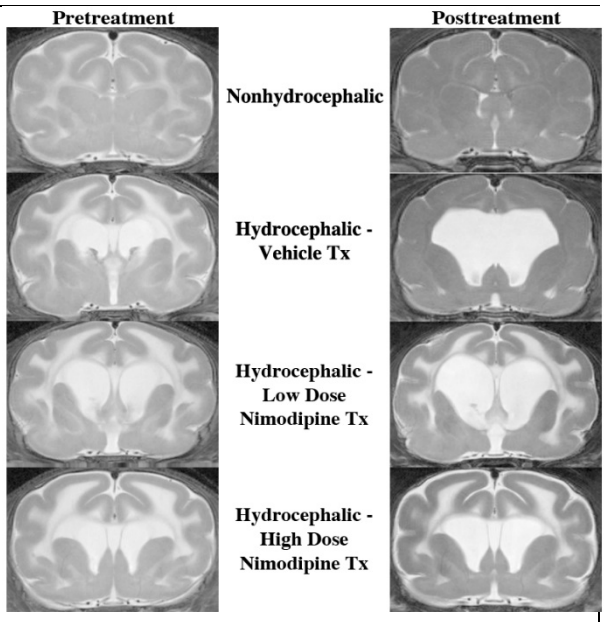

Figure 2: T2 weighted magnetic resonance frontal coronal images of the lateral ventricles of control kits and the three hydrocephalic treatment groups: sham, low dose, and high dose nimodipine, before and after drug treatment.

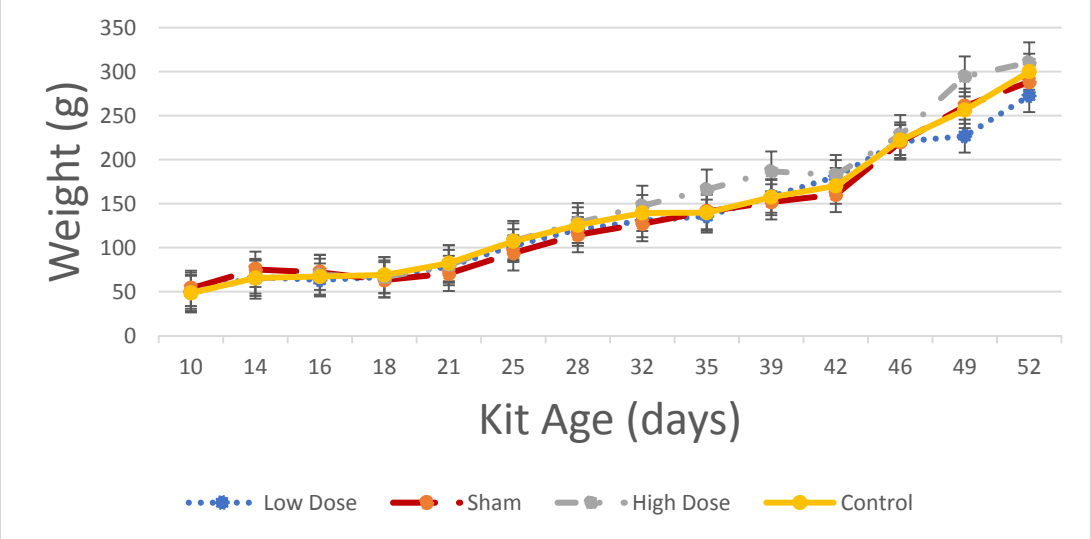

Figure 3: Line graph (mean± standard error) showing the weight trend of control animals as well as the three treatment groups: sham, low dose, and high dose nimodipine. Weights were taken daily but are shown only every 2-4 days in the above figure. No significant differences were noted between the groups.

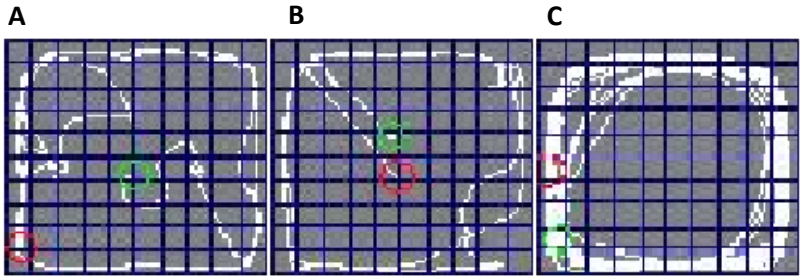

Figure 4: Tracking of ferrets' motion in the open field test using the HVS Image 2100 Plus Tracking system, showing the differences between a control kit (A), a moderately hydrocephalic kit (B), and a severely hydrocephalic kit with blindness (C). The first two are similar while the latter shows hyperactivity running around the edges of the open field area. All kits shown received low dose nimodipine.

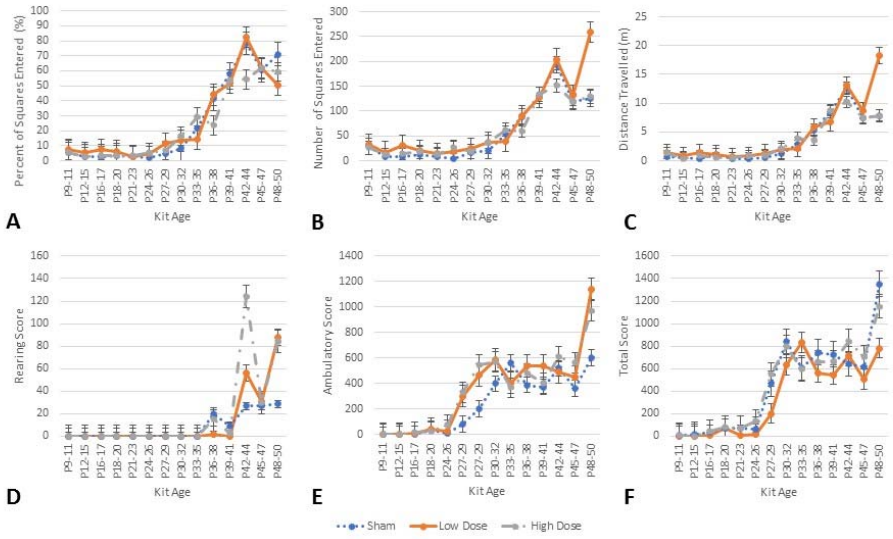

Figure 5: Behavioural scores of the three hydrocephalic nimodipine treatment groups presented graphically. Graphs A-C show the scores of the open field HVS Image 2100 Plus Tracking system: (A) percent of the total cells entered, (B) number of different cells entered, (C) the total distance covered. Graphs D-F show the scores of the open field Opto-Varimex 3: (D) the number of times the vertical (rearing) sensor was tripped, (E) the number of time the horizontal (ambulatory) sensor was tripped, (F) as well as the total number (vertical + horizontal) of sensor trips. Behavioural testing was done every three days starting at P9-11 and continuing until P48-52. In general, activity increased as the kits matured; however, there was no significant difference between the groups.

Table 1: Results of nimodipine treatment on hydrocephalic ferrets for control, vehicle, low dose and high dose treatment groups.

	Nonhydrocephalic	Hydrocephalic-Vehicle	Hydrocephalic-Low Dose	Hydrocephalic-High Dose
Sample Size	7	3	4	3
Lateral ventricle area index (2 days post kaolin / pre-treat)	0.036±0.005^	0.092±0.023	0.096±0.010*	0.064±0.022
Lateral ventricle area index (P29 / pre-treat)	0.023±0.004^	0.153±0.072	0.135±0.046	0.185±0.083
Lateral ventricle area index (P52 / post-treat)	0.27±0.003^	0.192±0.067	0.228±0.145	0.380±.204
Percent enlargement ventricles during treatment (%)		22.1±32.3	46.3±46.5	98.1±24.8
Rearing activity (beam breaks per 3 min) (P48-50 / post-treat)	119±11^	87.7±18.9	28.8±18.3*	84.3±52.7
Ambulatory activity (beam breaks per 3 min) (P48-50 / post-treat)	730±61	604±88	1143±615	971±225
Total activity (beam breaks per 3 min) (P48-50 / post-treat)	930±68	783±110	1353+670	1153±230
Percent cells entered – open field (per 3 min) (P48-50 / post-treat)	66.7±6.6	71.0±4.4	50.8±8.7	59.3±9.7
Number cells entered - open field (per 3 min) (P48-50 / post-treat)	132±15.1	126±12	259±146	129±17
Distance traveled - open field (m per 3 min) (P48-50 / post-treat)	8.37±1.01	7.77±0.79	18.3±12.3	7.84±1.21
Diminished vision / blind (P48-50)	0 / 0	0 / 1	0 / 0	1 / 2

* $p<0.05$ vs control kits, t-test

^$p<0.05$ vs hydrocephalic kits, t-test