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Continuous Monitoring of Changes in Plasma Nitrite Following Cerebral Ischemia in a Rabbit Embolic Stroke Model

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Abstract

In this proof-of-concept study, we investigated direct, continuous monitoring of plasma nitrite as an indicator of cerebral ischemia following clot embolization of rabbits via an indwelling carotid catheter. Two groups of rabbits were studied to compare the effects of embolization on nitrite levels. In the control group, blood was continuously obtained from a jugular venous catheter. The blood was immediately passed through an ultrafiltration filter; the filtrate was chemically reduced to convert free nitrite to nitric oxide (NO) and then measured using a NO-specific electrode. In the embolized group, after a baseline nitrite level was achieved, blood clots were injected into the brain via the carotid artery catheter, and then nitrite levels were continuously measured from jugular venous blood. The stroke group showed a significantly greater increase in nitrite as compared to controls ($p=0.017$). Using the area-under-the-curve (AUC) method, results reached statistical significance ($p<0.05$) within 3 min of embolization. In embolized rabbits, NO₂ levels increased $424\pm256\%$ compared to baseline. This study shows that nitrite can be measured immediately following a stroke and that our system measures nitrite independent of the extent of the stroke. This study provides evidence for the feasibility of using nitrite as a marker of ischemic stroke.

Keywords

Diagnostic biomarker; Nitric oxide; Ischemia; Plasma measurement; Validation

Introduction

Stroke is the third leading cause of death in the USA and is a leading cause of serious, long-term disability [1, 2]. Of the roughly 795,000 strokes each year, 200,000 are fatal and the 5-year survival rate of nonfatal strokes is only 50% [2]. The estimated direct US cost of stroke for 2009 is \$54.2 billion with the long-term costs for an individual estimated to be \$140,000 [3]. Ischemic stroke accounts for approximately 87% of all strokes, while the remainder are hemorrhagic strokes [2]. In over 60% of stroke patients, vascular thrombus deposition is responsible for the interruption of cerebral blood flow (CBF). Reduced CBF and severe oxygen deficiency lead to ischemia and eventually to behavioral and functional deficits,

morbidity, and mortality [4, 5]. Even in hospitals, where 6.5% to 15% of strokes occur (representing 47,000–119,000 patients), delayed detection, assessment, and treatment are common [6]. Key factors in predicting stroke morbidity and mortality are severity and time to treatment, with earlier treatment equating with improved outcome [7]. Since stroke symptoms can be ambiguous and many patients are alone, sedated, in surgery, or asleep during stroke onset, delays in recognition and assessment are common [8], thus significantly limiting the ability to provide timely therapy. A rapid monitoring technique to aid in the detection of ischemic stroke in humans may prove useful in determining a treatment regimen to promote functional recovery.

The role of nitric oxide (NO) in ischemia is widely accepted and is supported by a body of preclinical [9–13] and clinical [14] research. There is a significant increase in NO almost immediately after ischemic onset, making NO a biomarker for ischemia. NO is produced by rapid conversion of nitrite to NO by red blood cells under ischemic conditions (low pH, low partial pressure of oxygen) [15]. Conversion of nitrite to NO by erythrocytes can occur in as little as 30 s to several minutes [16]. Nitrite has been shown to be a circulating storage pool for NO [17, 18]. NO is also produced by the enzymes endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS) which are both activated in about 10 min [19]. Since NO is produced almost immediately by conversion of nitrite by red blood cells, and nearly simultaneously by both eNOS and nNOS [19], it is not necessary to discriminate between these potential sources of NO to detect ischemia in the rabbit model used in this study. In rats, NO_x rises in jugular venous blood immediately following a stroke induced by middle cerebral artery occlusion [10] and then returns to normal when ischemia is relieved. NO is linked to both focal and global ischemias [20]. In a rat model of complete global ischemia, NO decreases rapidly for 10 min and then increases rapidly [20]. Thus, this may differentiate global from focal ischemia. Clinical research shows that NO_x is also significantly elevated in cerebrospinal fluid following ischemic stroke [14].

We have chosen to measure plasma nitrite (NO_2) as a surrogate biomarker [21] for nitric oxide (NO), which is an indicator of ischemia. The majority of nitrite in human blood is transported within erythrocytes [22] and thus is not detected by our system which filters out erythrocytes and hemoglobin, so we are therefore measuring plasma nitrite or free nitrite. Factors favoring the conversion of intracellular (rather than free) nitrite to NO are the high concentration of intracellular hemoglobin [22] (about 20 mM), the large fraction of nitrite which is stored within erythrocytes, and the short diffusion distance for intracellular nitrite as compared to free nitrite. Conversion of intracellular nitrite to NO, followed by release from red blood cells, followed by rapid oxidation back to nitrite would produce an increase in free nitrite. Such a change would be favored under conditions of ischemia and would be detectable with our system.

Because NO is involved in numerous biological pathways, its specificity as a marker for cerebral ischemia will be dependent upon the NO concentration, kinetics, and localization [23, 24]. With five enzyme cofactors [25] for the NOS enzymes, it is clear that NO is tightly regulated by the body under normal physiological conditions. For example, several hours after stroke onset, inducible nitric oxide synthase (iNOS) is activated and generates relatively large amounts of NO. However, the rate of increase in NO_x levels generated by eNOS and/or nNOS (during ischemia) is from 10 to 50 times faster [10, 26] than the rate of NO_x generated by iNOS (during inflammation or infection), thus allowing the distinction between ischemia and inflammation or infection. Thus, even if an initial NO_x baseline rises due to inflammation, it is still possible to distinguish ischemic events superimposed on inflammatory processes, by continuous measurement of plasma nitrite.

In the present proof-of-principle study, we used a novel detection technique to measure free nitrite, in combination with an embolic stroke model [27] to determine the feasibility of using nitrite as a biomarker for acute ischemic stroke.

Methods

Figure 1 illustrates the concept of the plasma nitrite monitor. The system measures free nitrite levels in blood and may be used on a continuous basis, as in the present study. The sampling catheter extracts blood, which is pumped from either a test subject or a test tube through an ultrafiltration cartridge (Spectrum Laboratories, Rancho Dominguez, CA) to separate nitrite and other low molecular weight components from the larger components (e.g., erythrocytes, leukocytes, platelets, and larger proteins) with the latter going to waste, or potentially being reinfused into the patient. The cartridge consists of multiple polysulfone hollow-fiber membranes with a total surface area of 20 cm² and a 10kDa molecular weight cutoff (MWCO). A transmembrane pressure gradient is applied between the inlet and outlet of the filter, resulting in separation of the blood elements by molecular weight. The filtrate containing the free nitrite is then pumped from the filter and mixed with a solution of 0.1 N H₂SO₄ and 5% (w/v) potassium iodide. This process chemically reduces the nitrite to nitric oxide (NO). The NO solution is then pumped into a flowcell containing an NO electrode (amiNO-700, Innovative Instruments, Tampa FL) and then to waste.

Sensitivity and Linearity

The system has been calibrated over a NO₂ concentration range from 10 nM to 5 μM in diluted blood (one part blood to four parts normal saline), which surpasses the normal physiological range (typically 100 nM to 1 μM) [17]. The initial response time to a 1 μM bolus of NO₂ is 1–2 min. The system was also evaluated for possible interference from, and sensitivity to solutions containing 0.1 M dopamine and 0.1 M ascorbic acid (final concentrations) as well as electrical interference from high-frequency sources.

Rabbit embolic stroke model

Rabbits were supplied food (alfalfa cubes) and water ad libitum while under quarantine in an enriched environment for at least 5 days prior to experimental use. Surgery was done in a sterile controlled environment with a room temperature between 22.8°C and 23.2°C. Institutional Animal Care and Use Committee approved the surgical and treatment procedures used in this study. Extreme care prior to and postsurgery and embolization was used to minimize adverse effects in study rabbits. The system was used to continuously monitor nitrite in blood in (*n*=3) test rabbits (2–3 kg male New Zealand White). Cerebral ischemia was induced using the rabbit large clot embolic stroke model of Lapchak [27, 28] by injection of a clot through the carotid artery into the brain. Measurements were obtained from blood from a catheter placed in the rabbit's jugular vein. In the control group, the rabbits were not embolized. In both groups, the NO₂ levels were monitored in the ipsilateral jugular vein. Rabbits were anesthetized with halothane (5% induction, 2% maintenance by face mask); the bifurcation of the right carotid artery was exposed; and the external carotid artery was ligated just distal to the bifurcation, where a catheter was inserted anteriorly (toward the brain) into the common carotid artery and secured with ligatures. The incision was closed around the catheter with the distal end left accessible outside the neck; the catheter was filled with heparinized saline and plugged with an injection cap. Large emboli were prepared by withdrawing 1 to 2 ml of arterial blood from a donor rabbit. The blood was allowed to clot for 3 h at room temperature and then sliced into small cubes weighing approximately 3.2 to 3.5 mg (approximately 25 mm³). The cubes were suspended in phosphate-buffered saline containing 0.1% bovine serum albumin.

After the carotid catheter was placed, an ipsilateral jugular vein cutdown was performed. The proximal and distal portions of the vein were temporarily ligated with 4-0 silk suture. A 5 F catheter was introduced into the vein retrograde to the flow and tied off. The rabbit was then injected with 1 to 1.5 ml heparin (1,000 U/ml). Nondiluted blood was extracted using a pump via the jugular catheter through a KRII ultrafiltration system (Spectrum Labs, Rancho Dominguez, CA) using a 10kDa polysulfone filter with a surface area of 20 cm², at a flow rate of 0.6 ml/min. The ultrafiltrate solution was reacted with a molar excess of sulfuric acid (0.1 N) and potassium iodide (5% w/v) in order to convert the nitrite stoichiometrically [29] to NO, which was measured using an NO sensor (amiNO-700, Innovative Instruments, Tampa FL). Prior to taking measurements in blood, calibration was performed with 100 nM and/or 1 μ M NO₂, and then a reading using distilled water was taken to determine the background nitrite level. Sensor output was continuously recorded using a commercial software package (Innovative Instruments). Baseline measurements of NO₂ concentration were obtained until the NO₂ background appeared to have stabilized. Just prior to experimental embolization, the injection cap of the catheter was removed to allow the rabbit's blood to fill the catheter and wash out the heparinized saline. The line was then filled with heparin-free normal saline. A "clot cube" was then suspended in 0.5 ml of normal saline and transferred to the injection catheter hub with forceps and rapidly injected with 3 ml of saline flush, followed immediately by a second 3ml saline flush. Care was taken during both flushes to ensure that no air bubbles were present in the catheter or syringe. In this study, a second blood clot was injected in the same way 3–5 min after the first embolization. Monitoring was conducted for about 30 min, or until the animal was determined to be unable to donate additional blood. After embolization, the catheter was capped, ligated close to the neck, and the rest of the catheter and injection port were cut off. Prior to and following embolization, the change in plasma nitrite concentration from baseline was monitored continuously using the jugular venous NO₂ sensing system.

Histology

After all measurements were made, each rabbit was euthanized with 1 ml Beuthanasia-D and the brain examined grossly for infarcts or hemorrhages as described previously [27, 28] or fixed with 10% formalin and preserved for histomorphometric analysis. Specimens were evaluated histomorphometrically for infarct area by a veterinary pathologist (Comparative Biosciences, Sunnyvale, CA).

Statistical Analysis

Because these studies were a comparison of normal control rabbits to embolized rabbits, they were not done blinded. Based on literature [10, 12], we hypothesized that the rate of change in plasma nitrite levels, rather than an absolute level, provides a key indicator of ischemia. Every animal in the stroke group showed a positive slope in the graph of NO₂ concentrations with time that was greater than the slope in the control group. Therefore, the data were fit using a linear least squares approach, forcing the data through the origin to determine the best fit line for each data set. Next, these slopes were compared between test animals and the controls using a *t* test assuming a two-tailed distribution and equal variances.

Analysis of NO₂ changes as a function of time was also performed using an "area-under-the-curve" (AUC) method, where values above baseline are considered positive and those below baseline are negative. The initial baseline was determined by averaging the first (*n*=240) data points for each data set (the software collected two data points per second, so this represents 2 min). This initial baseline was then subtracted from each data point, and the data were integrated over time. For the stroke group, this integration began at the time the first clot was injected, while the integration for the control group started at the beginning of

the measurement. Finally, the areas (nanomolar*second) were compared between test animals and the controls at 1-min intervals using ANOVA followed by a post hoc *t* test.

Results

Equipment Calibration and Sensitivity Studies

Figure 1 shows a schematic diagram and a photograph of the benchtop prototype system for continuous monitoring of NO₂ in blood samples. In preliminary bench studies, this system was calibrated using pure NO₂ solutions over a range from 10 nM to 5 μM NO₂. Using NO₂-spiked samples of blood, a calibration curve over a range of concentrations (10 nM to 5 μM NO₂) was constructed (Figs. 2 and 3) that shows a linear correlation coefficient of $r^2=0.99$ for sensor output to NO₂ concentration from 0.1 to 5 μM and $r^2=0.99$ from 10 to 500 nM. Sensors were tested and determined to be insensitive to ascorbic acid and dopamine, each with final concentrations of 0.1 M, thus well above likely physiological levels. The system was found resistant to electrical fluctuations likely to occur in an operating room (OR) or an intensive care unit (ICU). (A 2W electrical source, ranging in frequency from 145 to 450 MHz, created measurable interference at 3 in., but not at 2 ft.)

Rabbit Embolic Stroke Study

The behavioral response to stroke ranged from head lean to severe response, and grossly visible infarcts or hemorrhages were only apparent in two of the three embolized rabbits. Hematoxylin and eosin (H&E) [30] staining revealed small infarcts in the cerebellum and brainstem, encompassing only 1% of the total area, in a third rabbit. Thus, all rabbits in this study had either infarcts or hemorrhages. Table 1 provides characterization of the brain damage and behavioral manifestations produced by embolization.

Nitrite Measurements

Figure 4 presents the data from each of the individual rabbit studies. Figure 4a from control rabbits shows that NO₂ levels were constant during the measurement period except for some detection system noise. The baseline nitrite levels were not significantly different between the two groups. In the stroke group, baseline NO₂ was 0.90 ± 0.36 μM, while that in the control group was 0.78 ± 0.66 μM ($p=0.8$).

However, as shown in Fig. 4b, there was a significant increase in nitrite in each embolized rabbit. There were quantitative differences between the extent of the increase that may be related to the extent of the stroke or presence of infarcts or hemorrhage in each of the embolized rabbits. For example, in Stroke 1, we visually identified hippocampal and thalamic infarcts and this rabbit had a large increase in nitrite. By comparison, Stroke 2, which only had a small cerebellar infarct detectable using H&E, the extent of nitrite increase was less, but nonetheless there was an increase in nitrite during the measurement period.

Derivative Method

The slope of the nitrite concentration vs. time for each animal is reported in Fig. 5, and a *t* test (two-tailed, assuming equal variances) shows a significant difference ($p=0.017$) between stroke and control animals.

Integral Method

Analysis of NO₂ changes as a function of time was also evaluated using an “area-under-the-curve” (AUC) or integral method, where values above baseline are considered to be positive and those below baseline are negative. This is a standard, robust, and rapid signal analysis method for obtaining clinically useful decision rules, such as is used in clinical near-infrared

spectroscopy (NIRS) monitors [31]. The monitor clearly and statistically differentiated between the NO₂ levels of the embolic and control groups at $p < 0.05$ (t test assuming equal variances and two-tailed distribution) in < 3 min (Fig. 6).

Histology

After nitrite measurements were made, each rabbit was euthanized and the brain examined grossly for infarcts or hemorrhages. Gross examination immediately following sacrifice showed clear evidence of infarcts and hemorrhages in two of three embolized rabbits, and in none of the controls. A brain from an embolized rabbit that had a behavioral reaction, but which showed no visible infarcts or hemorrhages on gross examination, was then paraffin embedded, serially sectioned, and stained using H&E. The particular rabbit was selected because it was the only animal that had no infarcts or hemorrhages visible on gross examination, thus it could provide an indication of the sensitivity of NO₂ release to infarct size. Microscopic changes observed were predominantly inflammatory and degenerative in nature; however, they were mild in severity and random in distribution. Histomorphometric evaluation showed minimal infarcts, with the largest amount in the cerebrum (1% of total brain area in the cross sections) and infarcts in the cerebellum and brainstem each comprised 0.1% by area. Since this rabbit showed a clear increase in NO₂ in response to the experimental stroke, this provides an indication that NO₂ release occurred with either just ischemia or due to small infarcts which were not grossly visible.

Discussion

This proof-of-concept study provides support for the use of continuous nitrite measurements as a dynamic biomarker to detect ischemia, infarcts, and hemorrhage following embolization. Typical biomarker measurements provide a snapshot of a patient's physiological status, which may have changed significantly between the time of measurement and the time at which therapy is initiated. In contrast, continuous biomarker measurement provides dynamic information about patient status. It may be of significant clinical utility to use a marker such as plasma nitrite to follow patient status on a continuous basis and then combine that with single point measurements of other biomarkers to improve sensitivity and specificity.

There are numerous technical considerations that must be evaluated when measuring dynamic changes in a proposed biomarker for stroke. One such consideration that must be addressed is system noise and the sensitivity of detection of the biomarker. Comparison of the differences in NO₂ levels between the maximum and baseline in the rabbit stroke group and the control group is suboptimal because noise plays such a key role in the outcome. Clinically, this method is undesirable because it provides slow response times in comparison to preferred derivative and integral methods. In this study, the absolute level method would have required analysis of data as much as 30 min after stroke onset. Moreover, initial baseline NO variability is expected between clinical subjects, since diet [32, 33], exercise [34], and anesthesia [35] may affect the nitrate, nitrite, and NO levels.

The integral (AUC) method reduces the influence of noise, providing a reliable result that is also faster than waiting for a maximum value to be reached. It is also less sensitive to noise than derivative (instantaneous slope) methods; thus, it can be the basis of a clinically useful decision rule. This method is in fact used clinically by NIRS monitors (Covidien). In the current study, a statistically significant result was reached within 3 min.

Ipsilateral jugular venous monitoring in rabbits showed a substantial increase in plasma nitrite concentrations following induction of embolic strokes. This study indicates that a rate of change in nitrite levels over time and not an absolute level can serve as an indicator

threshold for ischemia. Importantly, this increase occurred in rabbits independent of the severity of the stroke. In addition, the changes in nitrite concentration occurred whether or not infarcts or hemorrhages were observed grossly or microscopically. The sample size in this study is too small to draw any correlation between the severity of stroke, the size or location of an infarct, and the increase in nitrite signal, or the time lag between embolization and signal increase. In this particular group of three embolized rabbits, none had a large blood clot lodged in the middle cerebral artery. If that would have occurred, we would have expected a substantially larger number of infarcts or intracranial hemorrhages (ICH) with increased rabbit morbidity [27, 28]. Nevertheless, one brain was evaluated histomorphometrically, and was chosen since this brain had no grossly visible infarcts. Histomorphometry demonstrated that the infarct area was only 1% of the cerebrum area, which provides a preliminary indication of the sensitivity of nitrite measurements to cerebral ischemia. This suggests that plasma NO₂ is a sensitive marker even in cases where large infarcts were not observed.

Alternative efforts to monitor NO or its metabolites in blood in real time have been unsuccessful. An NO sensor placed directly on a catheter showed insufficient signal (only 2 nM) in blood [36], and continuous measurements of nitrite levels in pure solutions will not work in blood because of interference by hemoglobin [37]. Hemoglobin (~68 kDa MW) does not affect the measurements in this study because it is filtered out by a 10-kDa MWCO membrane. Measuring multiple markers simultaneously may ultimately improve sensitivity and specificity.

The rabbit embolic stroke model was developed and refined by Lapchak and colleagues to mimic human ischemic strokes. However, injection of a large blood clot into a carotid artery catheter system which is directed to the brain does not target a specific blood vessel, and as recently shown by Lapchak [27, 28], the severity of the stroke can vary from animal to animal, as does the extent of both ischemic damage and hemorrhagic damage [27, 28] just as in the clinical situation. In the present study, embolization resulted in both behaviorally and neurologically minor and moderate strokes. Despite this variability, an increase in plasma nitrite was observed in each of the embolized test animals, but not in the nonembolized control rabbits.

Another limitation to the study that must be mentioned is the blood volume required to measure dynamic changes in nitrite levels. In our study, because of the flow rate that was required, the monitoring period was limited to about 30 min because only 15 ml of blood could be drawn. With a lower flow rate, the sample extraction rate could be reduced and therefore the monitoring time could be extended. There are certain patient populations for whom it may be important to minimize sample volumes in order to avoid transfusions. In general, there would be a benefit to drawing as small a volume of blood as possible, in order to be able to maximize the monitoring period.

Summary and Conclusions

In conclusion, in this preclinical proof-of-concept rabbit embolic stroke study, we found an increase in NO where there were clinical signs of stroke even when histology showed only very small infarcts comprising less than 1% of the total brain tissue area. Importantly, this provides evidence suggesting that NO is a marker for either ischemic tissue or tissue with small infarcts or hemorrhages and that the sensitivity of the assay is high. These findings, in conjunction with the underlying physiological basis of NO biomarker biology, clearly indicate that continuous monitoring of NO₂ levels in the blood could be a valuable marker for stroke.

Changes in plasma NO₂ in the rabbit embolic stroke model indicate its potential use as a clinical diagnostic monitor for stroke. In future studies, the ability to detect events in the brain by monitoring via peripheral arterial blood or from a central venous catheter may widen the scope of clinical applicability. A low-cost, simple to use, minimally invasive continuous cerebral ischemia monitor will allow timely assessment of cerebral perfusion complications and timely application of appropriate therapy. Inhospital stroke may account for as much as 6% to 15% of all strokes. Many of these patients are in the OR or ICU and could potentially be monitored for early evidence of stroke using this approach. The ability to monitor cerebral ischemia could provide for a significant decrease in morbidity and mortality for these high-risk patients.

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Fig. 1.

Photograph and block diagram of prototype nitrite detection system. The sample is drawn either from a catheter (as in the present study) or from a test tube, pumped through an ultrafiltration membrane, where the filtrate is extracted. The filtrate is then mixed with a solution of sulfuric acid and potassium iodide to reduce nitrite (NO₂) to nitric oxide (NO). The nitric oxide is measured using an NO-specific electrode. Finally, the signal is sent to a PC for readout and signal processing

Fig. 2.
In vitro measurement of NO₂ in anticoagulated human blood from 100 nM to 5 μM.
Timescale of 200 s per division

Fig. 3. Correlation between nitrite concentration and signal. The graph shows a plot of signal in picoamperes (pA) vs. concentration of NO_2 (nanomolar) in heparinized human blood. The results are presented as mean $\pm$ standard deviation. Within the parameters of the test system, there is linearity of NO_2 between 10 and 500 nM ($r^2=0.99$)

A: Control Rabbit Measurement **B: Embolized Rabbit Measurement**

Fig. 4.
a Control rabbit measurement. **b** Embolized rabbit measurement. Original data traces for ($n=3$) stroke rabbits and ($n=3$) control rabbits; **a** shows raw data for the three control unembolized rabbits, and **b** shows raw data for three embolized rabbits. The black arrow in **b** shows the time of embolization (150 s) after a stable baseline was achieved. Note the sharp increase in NO_2 levels measured in embolized rabbits compared to the flat horizontal levels in the control rabbits. In embolized rabbits, the change in NO_2 levels extrapolated from the curves at 1,275 s is $424 \pm 256\%$ compared to baseline. There was a significant ($p < 0.05$) change in slope and an increase in NO_2 in rabbit 2 identified as Stroke 2 even though this rabbit had a small cerebellar infarct

Fig. 5.

Slope of NO₂ concentration vs. time. Slopes for each animal (in nanomolar per second) were determined by linear least squares method, forcing each data set through the origin. The stroke group was then compared to the control group, using a two-tailed *t* test assuming equal variances ($p=0.017$)

Fig. 6. Area-under-the curve (nanomolar*second) vs. time. The AUC for each group was calculated by determining a baseline (averaged over the first 2 min of measurement), and then subtracting this baseline from the data after clot injection. The data were then integrated over time for 1–5 min. Using ANOVA, with a post hoc *t* test, statistically significant differences were observed by the 3 min time point (* $p < 0.05$)

Table 1

Summary of behavioral responses and brain pathology

Rabbit	Behavioral response	Pathology
1	Head lean.	Visually identified hippocampal and thalamic infarcts.
2	Severe stroke response, with tremor and temporary loss of consciousness.	No infarct or ICH upon visual inspection. Presence of cerebellar infarcts using H&E staining.
3	Struggle and head lean.	Visually identified infarcts in cerebellum and thalamus. Hemorrhages in cerebellum, frontal cortex and hippocampus.