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Rat Endovascular Perforation Model

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Abstract

Experimental animal models of aneurysmal subarachnoid hemorrhage (SAH) have provided a wealth of information on the mechanisms of brain injury. The Rat endovascular perforation model (EVP) replicates the early pathophysiology of SAH and hence is frequently used to study early brain injury following SAH.

This paper presents a brief review of historical development of the EVP model, details the technique used to create SAH and considerations necessary to overcome technical challenges.

Introduction

At least three rodents (rat) models for studying early injury following subarachnoid hemorrhage are available. Two of these create SAH by injecting autologous blood directly into the cisterna magna (single injection model) or into the prechiasmatic cistern (prechiasmatic SAH)^{1, 2}. The third model creates SAH by perforating the intracranial bifurcation of intracranial artery (endovascular perforation; EVP model)³. Hence, in theory, EVP replicates the trauma experienced by the brain upon aneurysmal rupture. In addition, pathophysiological events observed in SAH patients during rebleed are also replicated by EVP model. For these reasons the EVP model is considered the closest representation of human SAH and is commonly employed to study early brain injury^{4–6}.

In the following the EVP model is reviewed. The purpose here is to provide some historical background on its development and on endpoints measured, to detail the technique used to create SAH and to present considerations necessary to overcome technical challenges.

Historical background of the model, evolution, and modifications over the years

Arterial puncture to create SAH was historically used in large animals such as cats, dogs, and monkeys. In 1979, Barry et al described the first model that performed arterial puncture in rats to create SAH⁷. The focus of Barry's study was to determine if delayed vasospasm develops and could be studied in small, less expensive animal species. Hence, in their first

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Compliance with Ethics Requirements

This article reviews but does not contain any studies with human or animal subjects.

paper Berry et al., defined the time line of development and resolution of vasospasm in rats. SAH here was created by opening the cranium at the midline to expose dura and advancing a tungsten wire into the lumen of the basilar artery. Bleeding was observed via craniectomy and a sponge was placed on the craniectomy site before closing. The craniectomy site was later reopened and enlarged and dura was removed for visualization and microscopic photographing of the basilar artery. The limitations of this model in the words of Barry et al are “because of potential trauma from repeated exposure of the basilar artery, data can only be obtained from one re-exposure in each animal. A second deficiency is the open skull which nullifies any effect that changes in intracranial pressure would have on spasm.”

In 1990, Kader et al., presented a modified version of Barry’s arterial puncture model ⁸. This modification used the same transclival approach (as ⁷) to puncture the basilar artery via tungsten wire, and measured CBF (Telencephalic blood flow) and ICP at 3, 7 and 7 days post SAH.

The modification that made EVP model incredibly appealing came in 1995 by Bederson et al, ³. Veelken and colleagues published a similar modification at the same time ⁹. Bederson’s modification creates SAH by puncturing the intracranial bifurcation of the internal carotid artery without opening the skull, and makes real time measurements of physiological changes which occur at arterial puncture. Bederson’s modification has made the EVP model, in theory, resemble the mechanism of acute aneurysm rupture. Hence, the EVP model in its present form is most commonly used to study acute injury after SAH.

Animals used

Age and gender—Rats used to create SAH are mostly young males; age 3–4 month old with weight of 300–400gm. This is despite the fact that incidence of SAH has preponderance in women and SAH victims on average are in the fourth or the fifth decade of life. The reasons for not including females and older animals are many. A major reason for not including females is to avoid hormonal variations due to the oestrous cycle. Moreover most research laboratories have traditionally used male rat for their experiments and addition of females requires them to revise anesthesia protocols because of gender-specific differences in sensitivity towards anesthesia ^{10–12}. Moreover, many general anesthetics are fat soluble and bind to fat cells, which changes their dose requirement and their pharmacokinetics, producing prolonged, less predictable effect. A careful modification of anesthesia protocol to accommodate for the increased body weight of older animals and of males versus females can help. It is interesting to note that female rats do not accumulate as much body weight as similar age male rats do; a 4 month old Sprague Dawley female weights approximately 250gm and a 4 month old Sprague Dawley male 350gms. Similarly, inclusion of urethane in the anesthetic regiment may help in blood pressure management. It is important to note that a recent study finds significant cross gender differences in early pathophysiology of SAH exist ¹³.

Animal strain differences—Strain differences can lead to considerable variability in the severity of early brain injury after SAH. We have noted that intensity of SAH is greater in Wister as compared to age-matched Sprague Dawley (SD) rats (unpublished data). This

difference is observed in acute physiology; Wistar males have greater ICP and BP rise at SAH and greater 24 hour post-hemorrhage mortality in Wistar males than age-matched SD males. The greater cerebrovascular collateral flow found in SD rats may have contributed to our findings¹⁴. A difference between the SD and Wistar strains in the intensity of injury after ischemic stroke has also been established¹⁵.

Variation in the gross anatomy of the internal carotid artery among different rat strains also occurs. Studies find a kink in the petrous segment of the internal carotid artery of Fischer-334 rats. This kink makes advancement of a filament towards the MCA difficult, and this rat strain is therefore considered unfit for MCA occlusion¹⁶. It will also be difficult to use Fischer-334 for EVP SAH since the filament may puncture the ICA at different sections and create inconsistent hemorrhages. This points to the importance of including strain, gender and age/weight matched shams in studies and, if possible, including multiple strains as well.

Endpoints for Therapeutic Studies

An experimental model can be used to study mechanism of injury and/or to determine efficacy of specific therapeutic interventions. Following are some therapeutic endpoints commonly studied when SAH is induced via EVP.

- a. *Vessel pathology*: Acute constriction of large and small cerebral vessels is present at one hour after SAH and widespread destruction of small vessel structure and disruption of microcirculation is present within the first 24 hours^{17–21}. Destruction of small vessel structure is observed as focal denudation, fragmentation and detachment of endothelium and loss of collagen IV; the major protein of basal lamina^{18–20}. Disruption in microcirculation is observed as wide spread perfusion deficits¹⁹ and increase permeability indicating breach of blood brain barrier^{14, 19–22}.
- b. *Inflammation*: Is studied as the activation of inflammatory molecules and cytokines^{23, 24}. Inflammation is observed as adhesion of platelet aggregates and neutrophil to the walls of small cerebral vessels^{25–27} and as increased immunoreactivity to nestin, ED1, OX6, intercellular adhesion molecule 1, and tumor necrosis factor²³. Prunel and colleagues observed that the inflammatory response after EVP SAH is less intense than that after injection of blood into the prechiasmatic cistern²³.
- c. *Cell death*: Widespread death of vascular (endothelial and smooth muscle) cells, neurons, astrocytes and oligodendrocytes occurs after EVP SAH^{14, 28–33}. We have found dead cells in brain parenchyma and in subarachnoid space at 10 minutes and in hippocampus at 24 hours after EVP SAH³¹. Others have noted that in EVP SAH cell death is present at 24 hours and persists for at least 7 days. Modes of cell death appear to include necrosis, apoptosis, and autophagy^{31–33}, and thus treatments that influence these pathways to reduce cell death are frequently studied^{34–36}.
- d. *Behavioral deficits*: Most studies evaluating behavioral deficits in EVP rats do so within 72 hours or at the most 7 days after SAH. Many of these studies, however do not monitor SAH physiology or include time-matched sham controls, which

makes interpretation of results difficult. Nevertheless, these studies find that behavioral deficits are present 24 hours after EVP SAH and are either substantially reduced or resolve by 7 days^{21, 28, 37, 38}. Deficits are found in animal's general behavior, sensorimotor function, coordination, and neurological functions (neuroscore).

Those few reports assessing long term (21 days) deficits in rats following EVP give conflicting results. Silasi et al. find no long lasting motor deficits and only minor cognitive impairment³⁹ and Sherchan et al., and Kooijman et al., report significant sensorimotor, cognitive, memory and motor deficits^{24, 40}. This difference may have resulted from two factors: 1) SAH intensity: whereas animals in Sherchan et al and Kooijman et al's study received moderate or severe SAH respectively, the severity of SAH that rats in Silasi et al., study received is not described, the early physiology of SAH was not monitored, and blood accumulation not studied. Hence, it could be that animals in their study experienced low intensity SAH.; 2) difference in tests for evaluating sensorimotor function: Sherchan used rotarod, Kooijman et al. used an adhesive removal task, and von Frey test and Salisi et al., used a tapered beam test, the forelimb asymmetry test, and the horizontal ladder task. Jeon et al., have recently detailed the neurobehavioral assessments found effective in experimental models of SAH⁴¹.

A word of caution on examining early behavior of EVP animals is that pre-operative anesthetics and post-operative analgesics can affect subsequent animal behavior; when animal behavior is examined as study end point, it is prudent to allow animal to recover from anesthesia fully before making judgments about behavioral deficits.

Delayed vasospasm—The rat EVP SAH model is not considered a standard model of delayed vasospasm⁴². Studies focused on delayed vasospasm use a double injection model instead^{42, 43}. Nevertheless, a number of investigators have used EVP to study cerebral vasospasm after SAH^{44–51}. It is noted that vasospasm after EVP SAH: 1) develops earlier than that after double blood injection; it is present at 24 hours and lasts for at least 72 hours; 2. in comparison, it is of lesser severity; and 3. It is present in intracranial artery, middle cerebral artery and basilar arteries^{43–45}.

Details of perioperative care, and monitoring

Anesthesia and Analgesia—Anesthesia, in the EVP model is required for pre and intra-operative procedures and an analgesic is used to relieve post-operative pain. The choice and dose of anesthesia are important considerations as anesthetics can interfere with brain injury mechanisms and contribute to study results. For example baroreceptor mediated rise in BP (Cushing) and spreading depression are two important component of brain injury after SAH are blocked by isoflurane and ketamine (Cushing)^{52, 53}. Combinations of anesthetic agents; anesthetic regimens, are often used to induce and maintain anesthesia in SAH animals. Anesthetic regimens are popular as they prolong anesthesia and reduce the dose requirement of a single agent that is usually associated with undesirable effects. However, anesthetic regimens if not carefully designed can exert their own additional effects. For example Hockel and colleagues have found that a combination of 2% isoflurane, 70% N₂O and 30% O₂ reduces CBF recovery in SAH rats⁵⁴.

Pre-operative anesthesia helps in intubation and placement of an animal on stereotactic frame. Anesthetic agents commonly used pre-operatively are injectables such as chloral hydrate, or a combination of ketamine- xylazine.

Intra-operative anesthesia is usually maintained via inhalation through a nose cone or via endotracheal tube. Anesthetic used include volatile anesthetics such as isoflurane, sevoflurane, enflurane and desflurane, non-volatile anesthetics such as nitrous oxide. In addition, injectable anesthesia; a combination of midazolam (2 mg/kg), medetomidine (0.15 mg/kg) and fentanyl (0.0075 mg/kg) has been used successfully for intubating and maintaining surgical anesthesia in rat during SAH surgery ⁵⁴.

Post SAH analgesia can be by bupernorphine (0.05mg/Kg, subcutaneous administration).

Perioperative care and monitoring

Surgical procedure in EVP model is complex and time consuming. Following life support ensures animal survival during and after surgery:

Respiratory support—Animal may stop breathing at SAH and expire. The reason for this spontaneous death is the rise in pressure on the central respiratory centers caused by extravasated blood, as reflected in raised ICP. Intubation or a nose cone can be used to ensure that animal continues to breathe (Figure-1 A, Figure-2 A).

Prior to SAH induction, monitoring arterial blood (femoral) for arterial oxygen tension (PaO_2), carbon dioxide tension (PaCO_2), and acidity (pH) ensures that animal is well ventilated (Table-1).

Maintenance of body temperature—Body temperature is monitored and maintained from the start of anesthesia until the rat recovers from it. Typically, a rectal probe is used for monitoring and a thermal blanket (such as a recirculating warm water heating pad) is used for maintaining body temperature during and after surgery. After surgery, when the rat begins to move, it is transferred to a heated cage. Unless the effect of temperature on brain injury is the focus of a study, rat body temperature is maintained at 37°C. Anesthesia can reduce rat's body temperature and an overhead lamp helps for its recovery and maintenance.

Pain management—The depth of anesthesia ensures that animal does not feel pain during surgery. That animal is not in pain can be monitored by corneal reflex and limb pinch as well as by monitoring of heart rate.

Fluid Support—Rat spends a long time on the stereotactic frame. Surgical procedure is time consuming and post SAH physiology monitoring increases this time on stereotactic frame even further. As the result rat, as it comes off the surgical table, is dehydrated and requires warmed saline or warmed lactated Ringer's solution (subcutaneous or intraperitoneal injection) to make up for the lost fluid. Some investigators replenish fluid during surgery through continuous infusion of ringer lactate via tail vein.

Detailed description of SAH induction

Rats are maintained under anesthesia throughout the surgical procedure (approximately 2.5 hrs).

Pre-surgical preparation

Anesthesia and Intubation—Animal is weighed and anesthetized (Ketamine- Xylazine; 50mg/5mg/Kg; IP). As sedation is achieved the dorsum of the head, the flexor surface of the neck, and the anterior and medial surfaces of the thigh and leg are shaved. Next, the animal is placed in a supine position; its neck is transilluminated; and a 3cm long PE-250 tube is inserted into the trachea (Figures 1A and 2A). The animal is now placed on a stereotaxic frame (Stoelting, Wooddale, IL). Body temperature is kept at 37 °C via rectal probe and heating pad (Harvard Apparatus Ltd., South Nantick, MA). The endotracheal tube is connected to an air supply and animal is ventilated on inspired isoflurane (1% to 2% in oxygen-supplemented room air; Figure 1A)

ICP Measurement—The rat is turned to a supine position and the superior surface of parieto-occipital suture line is located. The occipital muscles are retracted to expose the atlanto-occipital membrane. A burr-hole is drilled in the superior aspect of the occipital bone and a screw anchor (Bioanalytical Systems, Indiana) is placed. The atlanto-occipital membrane is pierced by a 23G needle and a cannula (PE-50) primed with normal saline is advanced through the atlanto-occipital membrane into the cisterna magna (Figure 1B). The other end of the cannula is attached to a pressure transducer and the ICP recording is observed. Fine adjustments (up and down movements) in the position of the cannula are made until nice undulating wave of intracranial pressure, indicating optimal cannula placement, is obtained. The cannula is secured in the cisterna magna first by tissue adhesive (vetbond, 3M St. Pauls MN) and then by acrylic cement and acrylic fluid (Lang Dental Co., Wheeling, IL).

CBF measurement—A midline incision is taken and the temporalis muscle is retracted. The periosteum of the temporal bone is scraped off using a jagged-tipped mill mounted on a drill, and Laser-Doppler probes (Figure-1C) are placed on the skull away in the territory of the middle cerebral artery but away from the large branches. At this point the laser Doppler probes are withdrawn and a moist piece of cotton gauze placed over the cranium to prevent the bone from drying.

The stereotaxic frame is now rotated keeping all recording devices in place so that animal is on its supine position. The frame is kept in this position for the reminder of surgery.

MABP measurement; femoral cannulation—An incision is made over the central portion of the thigh and leg. Tissue is dissected to visualize till the femoral neurovascular bundle(Figure-1D). The femoral artery is ligated distally with 4-0 silk. A vascular clip is placed proximally on the femoral artery to occlude the lumen temporarily. A small incision is made on the femoral artery, between ligature and vascular clip and a PE-50 tube attached to a pressure transducer is advanced into the artery such that it remains open towards the arterial lumen. The cannula is secured by with a 4-0 silk ligature.

For blood gas measurement—Femoral blood (50ul) is withdrawn in a heparinized syringe and analyzed for blood gases. Ventilation is adjusted as required to maintain the arterial blood gases.

Neck Dissection—A midline incision is taken; the strap muscles of the neck are retracted laterally. The tendon of the digastric muscle is identified and a retractor is wound around it. This is the highest point of reference, in neck dissection. The external carotid artery (ECA) is located (medial to digastric tendon) and a 4-0 silk ligature is placed around it (ligature-1). The hyoid cartilage is identified, and cleared from its muscular attachments. The ECA is traced distally up to its origin from the common carotid artery (CCA). The fascia is cleared until the ECA and the internal carotid artery (ICA) are isolated and free from all attachments. A 4-0 silk ligature with a 15 cm tail is tied below ligature-1 (ligature-2). The ECA is cut between ligatures 1 and 2 and the lower free end is retracted downwards so that it is aligned with the ICA into a straight line (Figure-1E). Two small ligatures are placed loosely on the ECA, between the retracted portion of ECA and the bifurcation of the CCA.

SAH induction

The Laser Doppler probe is positioned on the skull and baseline CBF, MABP and ICP are collected (usually a 20 minute pre SAH recording). A small incision is taken on the ECA distal to the 2 small loose ligatures placed earlier. Using forceps a 3-0 prolene suture (4 cm) is fed into this vessel. As the suture enters the ICA the 2 small loose ligatures are fastened over the endovascular filament. The distal end of the suture is fed into the ECA, till approximately 37 mm is in the artery and resistance is felt; this indicates that suture has reached the intracranial ICA bifurcation. At this time the suture is advanced further to puncture the artery and is quickly withdrawn to minimize any ischemic process which might be superimposed on the subarachnoid hemorrhage. Rises in intracranial pressure (ICP) and in mean arterial blood pressure (MABP; if SAH is severe) and a fall in cerebral blood flow (CBF) register SAH (Figure-1G).

Post operative considerations

The animal is kept on the frame and ventilated for at least 10 minutes after which anesthesia is reduced and, as animal wakes up and starts fighting with the intubation line, it is taken off the frame without removing the endotracheal tube, and is transferred to a heated cage and provided fluid support (see above). Most animals (approximately 90%) extubate themselves within hours; however, few need help from surgeon for tube removal. Extended (to 3–6 hours post SAH) ventilation with or without hourly readjustment to maintain normal blood gas can be used to increase animal survival after EVP SAH ^{54, 55}.

Sham controls

Controls for EVP models are sham-operated animals matched in age, gender and time. Sham surgery includes all steps that are carried out for SAH surgery, except for perforation of the internal carotid artery; the suture is advanced, and then retracted and removed upon reaching the intracranial ICA bifurcation ¹⁹.

Technical considerations

The surgery used to create SAH in EVP model is complicated and challenging. Hence, the learning curve for a new person is steep and it takes time before he/she produces animals useful for data collection. Moreover, due to the complexity of the surgery, intraoperative animal mortality can be extensive^{56, 57}. The following considerations have reduced early mortality in our laboratory (immediate death=0; 24 hour mortality: 26%) and shorten the learning curve of a new surgeon:

Intubation to reduce acute deaths

During surgery intubation maintains airway patency and can be the route of delivering volatile anesthesia. *At SAH* as increased ICP depresses brainstem respiratory centers, it ensures that the animal does not spontaneously stop breathing and expire (Figure 2A). Insertion of an endotracheal tube is not a trivial matter as if not properly done it can cause trauma to the larynx or surrounding structures or cause esophageal intubation. Moreover, multiple attempts to intubate are not recommended since, if the first few attempts fail, reflex closure of the glottis occurs and makes subsequent intubation attempts difficult if not impossible. The size and length of the endotracheal tube depend upon the weight of animal. Several devices and kits are now commercially available that can help in intubation. Signs of successful intubation include: fog (respiration) build up in the endotracheal tube upon each breath and synchronization of the animal's chest movement with the ventilator. A bulge in the animal's abdomen is a clear sign that the tube is in the esophagus and not in trachea.

Force of ICA perforation

Since EVP is a non-craniotomy model, the surgeon pushing the suture to perforate intracranial ICA bifurcation does so blindly and relies on the sensation of arterial perforation, felt through the inserted suture, without being able to see it happen. If an ICP rise is not observed in the real time recording, the surgeon may continue to advance the suture even after the artery is perforated, leading to multiple perforations and stabbing the brain tissue⁶. Moreover if the force used for ICA perforation is excessive then the whole arterial tree moves and leads to subdural hematoma and not to SAH⁵⁶. Subdural hematoma, if expected can be identified by the pattern of ICP decline after initial rise (immediate fall to baseline) and by lack of blood accumulation around circle of Willis. A stab wound, if suspected, can be confirmed by brain histology (Figure 2B). Brain stabbing can be prevented by attempting the arterial rupture once only and withdrawing the suture as ICP begins to rise; real time measurement.

Park and colleagues have presented an important modification to prevent subdural hematoma and associated mortality in EVP SAH⁵⁶. In this modification, a hollow polytetrafluoroethylene tube is first advanced through the intracranial ICA until it passes the ICA bifurcation and enters the proximal anterior cerebral artery (ACA), and SAH is then created by advancing a tungsten wire through the tubing until the proximal ACA is punctured.

Establishing and controlling hemorrhage severity

SAH severity is defined by peak ICP rise and the volume of subarachnoid blood; greater the ICP peak and subarachnoid blood greater the hemorrhage severity. As ICP peak and subarachnoid blood are the product of force used to perforate ICA hemorrhage severity, their reproducibility in EVP model is poor. A controlled and reproducible SAH severity reduces variation in the data which would be large if animals with varying SAH severity were grouped together. One way of controlling hemorrhage intensity is keeping size of mono filament used to create SAH constant (mono filament size is directly proportional to SAH severity),^{58, 59}. Nevertheless lack of hemorrhage reproducibility in EVP model makes it absolutely necessary that hemorrhage severity for all animals included in a study is established. Several methods of establishing hemorrhage severity are currently in use. These include; (1) *measurement of peak ICP rise*¹³; (2) *measurement of CBF recovery at 60 minutes after SAH*; SAH is rendered lethal and animal has little chance of 24 hr survival if recovery is less than 40%¹⁷; (3) *volume of subarachnoid blood*: greater the blood volume greater the SAH severity^{13, 48, 58}; and (4) 24 hr neurological score²⁴.

Conclusion

The rat EVP model not only replicates physiological events that follow SAH but also replicates the trauma felt by brain upon artery rupture. Hence this model, in theory is the closest imitation of human SAH. It is more suited and more frequently used for studying early brain injury than the delayed complications after SAH.

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Figure 1. SAH surgery

A shows rat on a stereotactic frame with endotracheal tube attached to the ventilator for respiratory support. B shows ICP cannula placement into the cisterna magna. C shows scrapping (thinning) of preosteum for placing laser Doppler probe. D shows femoral bundle; note femoral nerve (FN), femoral vein (FV) and femoral artery (FA). Femoral artery will be cannulated for mean arterial blood pressure measurement. E shows common carotid artery (CCA) and external carotid artery (ECA). F shows retraction of ECA by a silk suture and alignment with the ICA. G shows typical online real time recording of SAH event.

Figure 2. Considerations for SAH surgery

In A note transillumination of neck for placement of endotracheal tube (ETT). In B note a stab wound resulting from stabbing of the brain tissue by suture used for ICA perforation.

Table 1

Physiologic parameters of Rat used

Parameter	Common use
Commonly used strains	Sprague Dawley and Wister
Gender	Usually males but females have been used
Body weight	350–400 gm
Body Temperature	37 ± 0.5 °C
Baseline Physiology	
MABP	90 ± 1 mmHg
ICP	2.5 ± 0.2 mmHg
HR	250–493 bpm
Blood gases:	
pCO ₂	40 mmHg
pO ₂	140 mmHg
Ph	7.4

Table 2

Pathophysiologic parameters to be expected

Parameter	Effect	Time from SAH
Physiological changes:		
ICP	Increase	Seconds
MABP	Transient increase	Second
CBF	Fall	Seconds
CPP (MABP-ICP)	Fall followed by partial or complete recovery	Seconds
Mortality	Approximately 30%	24 hr
Small vessel response:		
Acute	Constriction	Minutes
Delayed	Not known	Not known
Large vessel response:		
Acute	Constriction	Minutes
Delayed	Constriction	24–48 hrs
Cell death:		
Endothelium	Present	Minutes
Neuron	Present	Minutes
Neurological deficits	Present	Days

Table 3

Advantages/Limitations/Disadvantages

Advantages	Limitations
Replicates the mechanical trauma experienced by the brain and cerebral vasculature	Requires extensive complicated surgery
Replicates events observed during rebleed in SAH patients: <ul style="list-style-type: none"> • Rapid ICP and MABP rise and CBF and CPP fall • Blood accumulation into subarachnoid space 	Poor control of bleeding and hemorrhage severity
Allows real time recording of early physiological changes after SAH	Lacks proper control
	High mortality