Bilirubin as a cerebrospinal fluid marker of sentinel subarachnoid hemorrhage: a preliminary report in pigs

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Object. A model of subarachnoid hemorrhage (SAH) in pigs was developed to investigate bilirubin concentration in cerebrospinal fluid (CSF) as a potential marker of sentinel SAH.

Methods. Seven male Yorkshire pigs received a 250-µl injection of either whole autologous arterial blood (four animals) or isotonic saline (three animals) into the cisternae magna in an effort to produce volumetrically a model of sentinel SAH and a control injection model, respectively. Cerebrospinal fluid volumes of 100 µl were then collected from both the lumbar cistern and cisternae magna at 2-hour intervals for a total of 24 hours postinjection. The CSF was then tested for bilirubin.

Mean concentrations of bilirubin (± standard deviation [SD]) obtained from the lumbar cistern 24 hours following the injection of blood or saline were 4.38 ± 1.04 µM in the SAH animals and 1.02 ± 0.05 µM in the controls. At 24 hours postinjection, mean concentrations (± SD) of cisternae magna bilirubin were 7.29 ± 1.33 µM and 1.33 ± 0.14 µM in the SAH animals and controls, respectively. In the SAH group, both the lumbar cistern and cisternae magna bilirubin concentrations differed significantly from baseline values 12 hours following SAH.

Conclusions. Elevated concentrations of CSF bilirubin can be detected following a low-volume SAH, and the production of bilirubin occurred over a predictable time course. Twelve hours after hemorrhage, an elevated CSF bilirubin concentration was an indicator of hemolysis occurring in the subarachnoid spaces. The presence of bilirubin in CSF is a potential marker for differentiating SAHs from traumatic lumbar punctures in humans.

Key Words • bilirubin • lumbar puncture • subarachnoid hemorrhage

Patients with headaches account for 1 to 2% of visits to emergency departments in the US. Within that population 4% of patients are eventually found to have a nontraumatic SAH.2,3,10,11 In those instances in which a large hemorrhage occurs, the clinical and radiographic profile of a patient typically renders an obvious diagnosis. Nevertheless, the literature reports a seemingly high incidence of initial misdiagnoses among patients with SAH. Recently, Edlow and Caplan1 estimated the rate of misdiagnosed aneurysmal SAH to range from 25 to 51%, with a mean delay of 6 days to establish the correct diagnosis.10,16,19 The ramifications of this delay include vasospasm, hydrocephalus, rebleeding, and death. Mayer, et al.,16 reported that 91% of patients with aneurysmal SAH had an excellent or good outcome when their disease had been correctly diagnosed initially and they had presented with good SAH grades (Hunt and Hess Grade I or II). In contrast, only 53% of patients whose original diagnosis had been incorrect had an excellent or good outcome, despite having presented with good SAH grades (Hunt and Hess Grades I or II).

A possible explanation for this rate of misdiagnosis is a fundamental shortcoming in the diagnostic paradigm; specifically, this relates to the interpretation of the LP results. The Stroke Council of the American Heart Association strongly recommends performing LP in patients with suspected SAH and nondiagnostic CT scanning results.12 The primary purpose of the LP is the detection of red blood cells in the CSF; however, an SAH must be differentiated from a traumatic LP, which occurs in up to 20% of the spinal punctures performed.13,14 It is, however, our contention that none of the currently available methods of CSF analysis can reliably distinguish an SAH from a traumatic LP. This dilemma is especially relevant in patients with sentinel SAH and has led to a high incidence of initial misdiagnoses in these patients.

The diagnostic paradigm for SAH can be completed using a reliable CSF marker specific for SAH. This marker must be consistently able to differentiate an SAH from a traumatic LP. We hypothesized that the identification of an elevated bilirubin contentration in the CSF can provide a sensitive, and potentially rapid, mechanism to detect a small sentinel SAH. With this in mind, we developed a model of sentinel (low-volume) SAH in pigs to measure CSF bilirubin as a means of both identifying SAH and excluding the diagnosis of traumatic LP. An integral component of our hypothesis was that the hemorrhage, hemolysis, and hemoglobin degradation after SAH leads to the production of bilirubin, whereas a traumatic LP is associated with relatively little bilirubin. We also demonstrated the timing and processes involved in the production of bilirubin in CSF following a sentinel SAH in the pig.

Abbreviations used in this paper: CSF = cerebrospinal fluid; HO-1 = heme oxygenase-1; LP = lumbar puncture; SAH = subarachnoid hemorrhage; SD = standard deviation.
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Materials and Methods

Bilirubin Assay

Total bilirubin was assayed using a method based on those developed by Michaelsson and Nosslin and adapted for use in a microtiter plate by a few of the authors (G.J.P.G. and J.F.C.). Briefly, samples were treated with a caffeine/benzoic acid reagent to make all of the bilirubin available for the diazotization reaction. A diazo reagent was added to yield a visible compound. This color change minimizes hemoglobin interference. Bilirubin concentration was determined using colorimetric analysis, by comparison with a reference curve.

In Vivo Experiments

Seven male Yorkshire pigs received a 250-μl injection of either whole autologous arterial blood (four animals) or isotonic saline (three animals) into the cisterna magna in an effort to produce volumetrically a sentinel SAH model or a control injection model. This volume was chosen because a 250-μl hemorrhage in a pig with a total CSF volume of 20 ml is equivalent to a 2-ml hemorrhage in a human with a 150 ml CSF. Although exact measurements are not possible, the volume of a sentinel SAH in a human was estimated to be 2 to 5 ml. Therefore, a 2-ml SAH in a human represents 1.3% of the total CSF volume (2 ml blood/150 ml CSF = 1.3%); this is equivalent to a 266-μl hemorrhage in a pig (pig CSF volume 20 ml × 1.3% = 0.266 ml). We chose a minimal amount of blood for injection to test the sensitivity of our experiment, and we rounded the 266-μl volume to 250 μl for convenience.

The animals were 2-month-old male pigs weighing 16 to 20 kg. All animals were initially anesthetized using intramuscularly delivered ketamine (30 mg/kg) followed by intravenously administered pentobarbital (35 mg/kg) via an ear vein. After inducing anesthesia, pigs were intubated with a cuffed endotracheal tube per os and received mechanical ventilation through a mechanical respirator (supplemented with 1 L/minute O2). In one animal, endotracheal intubation was unsuccessful and a tracheostomy was performed. Using a cut-down in the right groin, the femoral artery and vein were accessed. An arterial catheter provided a means of recording blood pressure and heart rate and obtaining blood samples (0.5 ml) for respiratory blood gas analysis, acid-base status, and hematocrit acid-base and respiratory gas analyzer (model #168; Ciba-Corning Diagnostics Corp., East Walpole, MA). Ventilatory adjustments were made accordingly to maintain normal blood gas parameters. Pentobarbital was infused continuously via a femoral vein catheter at a rate of approximately 20 mg/kg/hr throughout the remainder of the experiment to maintain a constant level of anesthesia. The limb-withdrawal response to painful pinch and the monitoring of arterial blood pressure and heart rate were used to determine the depth of anesthesia. Body temperature, measured using a rectal thermometer probe, was maintained at 38 ± 0.5°C with the aid of a warm-water blanket and a portable thermal fan.

To access the lumbar cistern, a dorsal midline incision exposed the spinous processes of the lower lumbar spine and the upper sacrum. A 22-gauge spinal needle was inserted into the interlaminar space (either L4-5 or L5-S1) to the dura mater. The purpose of the surgical exposure was to facilitate a nontraumatic LP. Spinal fluid was collected, centrifuged, and labeled as time zero. The spinal needle’s stylet was reinserted, and the needle was left in place for the remainder of the experiment. Cerebrospinal fluid volumes of 0.1 ml were collected from the lumbar cistern at 1- to 2-hour intervals for a total of 24 hours postinjection of blood or saline (see later). Immediately after collection, CSF was centrifuged and stored at −30°C until analysis.

Each animal’s cisterna magna was accessed using a midline incision at the craniocervical junction. An 18-gauge spinal needle was then inserted through the dura mater to reach the cisterna magna. The initial CSF was collected, centrifuged, and stored at −30°C. Injections of either 250 μl whole autologous arterial nonheparinized blood or 250 μl of 0.9% sodium chloride were administered via the spinal needle. The autologous blood had been collected from the femoral arterial catheter and was injected immediately after its collection. The spinal needle’s stylet was reinserted, and the needle left in place for the remainder of the experiment. To facilitate the flow of CSF to the lumbar cistern, each animal’s head was elevated using the reverse Trendelenburg position to an angle of 20°. Cisternal CSF samples (100 μl) were serially collected over 24 hours, immediately centrifuged, and stored at −30°C for assay.

Four animals made up the experimental arm, each with a 250-μl volume of blood delivered to the cisternae magna. Three control animals received an injection of 250 μl isotonic (0.9%) saline into the cisternae magna. As an extra control measure, a deliberately traumatic tap was performed in the control animals at the end of the experiment (time = 24 hours). Cerebrospinal fluid was collected after the traumatic tap, centrifuged, and stored at −30°C. Immediately following the 24-hour collection, both control and SAH animals were humanely killed.

Statistical Methods

Statistical analysis of the data proceeded from simple descriptive to complex inferential. First, a regression curve was fitted to the bilirubin data as a function of time. This was performed separately for cisternae magna and lumbar cistern data. The 95% prediction limits were obtained around the fitted curve, and the upper 95% confidence limit of the control group mean was compared with the lower 95% predicted limits for the curve. A probability value of 0.05 or less was considered statistically different. The earliest time point at which the two limits separated from each other (that is, they did not overlap) was declared to be the time (in hours) since the beginning of the experiment when the bilirubin levels in the SAH group significantly exceeded control group values. This analysis was also confirmed using a standard analysis of variance procedure, in which the control group least-square means were compared with the SAH group least-square means.

Results

In Vivo Experiments

In this model of SAH in pigs, results of sequential analysis demonstrated consistent and reproducible elevations in bilirubin concentrations in lumbar cistern and cisterna magna CSF obtained from the SAH animals. Following an initial delay of 12 hours, bilirubin concentrations in both the lumbar cistern and cisterna magna CSF differed significantly from baseline values in the SAH animals. Conversely, concentrations of bilirubin in the CSF of control animals did not rise significantly above baseline values. Figure 1 shows the time course of bilirubin production in our model. The mean concentrations of bilirubin (± SD) obtained from the lumbar cistern 24 hours following the injection of blood or saline were 4.38 ± 1.04 μM in the SAH animals and 1.02 ± 0.05 μM in the control animals, respectively. At 24 hours postinjection, mean bilirubin concentrations (± SD) in the cisterna magna were 7.29 ± 1.33 μM and 1.33 ± 0.14 μM in the SAH animals and the controls, respectively. The earliest time point at which control and SAH group bilirubin values did not overlap was determined using the standard analysis of variance procedure in which the least-square means were compared. The mean values of lumbar cistern and cisterna magna data first significantly differed at 12 hours (p = 0.05 for lumbar cistern, and p = 0.007 for cisterna magna).

Table 1 features bilirubin concentrations found in the pig, including the mean concentrations at the zero time point (control CSF) and in CSF obtained during a deliberately traumatic tap at the 24-hour time point in control animals.

Discussion

Bilirubin Production

We presented evidence that elevated bilirubin concentra-
 traditions in CSF is a marker of SAH. Additionally, we demonstrated the absence of bilirubin production following a control injection of saline. The production of bilirubin followed a reproducible time course that was likely attributable to known degradation processes of blood-derived heme. Indeed, the constituents of these degradative processes have been modeled in our laboratory by using an in vitro system (Pyne-Geithman, et al., personal communication), and our findings support the differential properties of CSF bilirubin concentrations in the context of SAH compared with traumatic LP.

Bilirubin production is a time-dependent process beginning after the introduction of blood into the subarachnoid space and the subsequent induction of the enzyme HO-1. This enzyme is responsible for the conversion of hemoglobin to biliverdin, which is then quickly converted to bilirubin. Furthermore, HO-1 is membrane-bound and found within the choroid, arachnoid, and glial cells. Therefore, once CSF is removed from the basal cisterns (and therefore the HO-1 activity), the production of bilirubin in this fluid essentially ceases. In our experiment, statistical significance for increased bilirubin concentrations was achieved 12 hours following hemorrhage and maintained over 24 hours in both the lumbar cistern and cisternae magna groups. Bilirubin concentrations in the CSF from the cisterna magna exceeded those from the lumbar cistern in all animals; this was likely the result of the recumbent position of the animal and the fact that the cisterna magna CSF was sampled from the site of hemolysis.

If differential CSF bilirubin analysis were extended to clinical application, the observed 12-hour time lag would be complementary to the ultrasensitive phase of computerized tomography scanning, which lasts up to 12 hours. In fact, the time-course of bilirubin production is precisely the value of this diagnostic modality. It is also important to note that there was no significant production of bilirubin in hemorrhagic CSF following the traumatic LP model (see Table 1). Thus, blood introduced during a traumatic tap would not have sufficient time for the catabolic conversion to bilirubin.

Traditional CSF Analyses

Although the role of CSF analysis in the diagnostic para-

digm is not novel, our ability to differentiate between an SAH and a traumatic LP is an important development. Currently, none of the available methods of CSF analysis provide a robust technique for distinguishing an SAH from a traumatic LP. This shortcoming is especially unfortunate in patients with sentinel headaches. Note that these headaches are thought to be caused by small warning leaks from an aneurysm and occur in 15 to 40% of patients. Although the diagnosis of sentinel hemorrhage is crucial, it represents a considerable diagnostic challenge and contributes substantially to the quoted rate for the misdiagnoses of aneurysmal SAH. A possible explanation for the rates of misdiagnoses is a fundamental deficiency in the interpretation of LP results.

The classic methods of CSF analysis, such as diminution in red blood cell count between serial collection tubes, have long been thought to preclude SAH. Data from Buruma, et al., have been frequently cited as the basis for the assertion that the serial tube method is a reliable predictor of traumatic LP. A review of this article revealed that only two of the 25 study patients with intracranial hemorrhage actually had an SAH. Furthermore, Buruma, et al., stated that “although there is a significant difference between the two groups (patients with hemorrhage and those without), no proper distinction could be made for the individual case.” Additionally, the serial tube method will not indicate when a true SAH has occurred in the presence of a traumatic tap. Alternatively, xanthochromia is touted as being indicative of an SAH. This technology uses the yellow discoloration of CSF supernatant to indicate the presence of bilirubin and an SAH. Currently, visual and spectrophotometric xanthochromia are used as indicators of CSF bilirubin content. Each modality has substantial limitations. The method of visual inspection lacks both objectivity and sensitivity, and some authors have reported an accuracy of only 50%. Alternatively, spectrophotometric analysis has limitations that have prevented its widespread application, in particular, the considerable overlap in the absorption of hemoglobin and bilirubin. In a recent survey of 3500 US laboratories, 1947 (99.7%) of 1952 respondents reported using visual inspection to detect xanthochromia. Our study revisits the concept of xanthochromia and directly measures CSF bilirubin.

A Model of Sentinel Hemorrhage

Two fundamental aspects of this model of SAH require further discussion. First, the volume of SAH delivered to
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an animal was volumetrically equivalent to a sentinel SAH in humans. With volumetric CSF conversions, a 250-μl SAH in a pig is comparable to a 2-ml SAH in a human. This implies that our model can sufficiently identify a clinically small SAH at a clinically important time after sentinel hemorrhage. Second, the animals used were juvenile pigs (mean age 2 months) and had levels of serum and CSF bilirubin that were elevated compared with those in healthy adult pigs; the studied pigs had a mean serum bilirubin of 4.5 mg/dl (healthy adult pig 0.4–1 mg/dl) and a baseline CSF bilirubin of 1.5 mg/dl (see Table 1). In adult humans, normal serum bilirubin levels are 0.4 to 1 mg/dl and the CSF contains no bilirubin. Although this may make a quantitative comparison of these data to human patients difficult, we believe that it is clinically important to consider increased bilirubin as an indicator of sentinel SAH.

Conclusions

In the model featured in this study, the measurement of CSF bilirubin, if collected 12 hours after the onset of symptoms, can distinguish between an SAH and a traumatic LP. Furthermore, bilirubin production occurs over a predictable time course that can be detected after a low-volume SAH. Effectively, the presence of elevated bilirubin concentrations excludes the diagnosis of a traumatic tap and allows for the diagnosis of an SAH (12 hours earlier). Clinical translation indicates a role for the measurement of CSF bilirubin in patients with a suspected aneurysmal SAH who present more than 12 hours after the onset of headache and who have a nondiagnostic computerized tomography scan. A rapid, quantitative analysis for bilirubin as a point-of-care technique will enhance the diagnostic paradigm for SAH and allow physicians to direct better patient diagnostics and treatment.

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References


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