1. **Abstract**

Subarachnoid hemorrhage (SAH) is a stroke with high rates of mortality and morbidity. SAH-induced cerebral vasospasm can lead to ischemic injury or death and is a common complication of SAH. Recently there has been an accumulation of emerging evidence that oxidation of heme-derived bilirubin into bilirubin oxidation products (BOXes) may be involved in cerebral vasospasm. BOXes are produced by the oxidation of bilirubin yielding a mixture of isomers: 4-methyl-5-oxo-3-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX A) and 3-methyl-5-oxo-4-vinyl-(1,5-dihydropyrrol-2-ylidene)acetamide (BOX B). BOXes have been a subject of interest in the neurosurgical and neurological fields for several years because of their purported correlation with and/or role in subarachnoid hemorrhage-induced cerebral vasospasm. We believe that it is critical to understand the chemical and biochemical environment in the hemorrhagic spinal fluid after SAH that leads to the oxidation of bilirubin. There is a growing body of information concerning their putative role in vasospasm; however, there is a dearth of information concerning the chemical and biochemical characteristics of BOXes.

2. **INTRODUCTION**

There is extensive study of the oxidations of unsaturated lipids and proteins in biological systems, including the resultant compounds produced (1, 2, 3, 4). This includes production of reactive oxygen species (ROS), free radicals, and enzymatic oxidations with single electron oxidations. The role and toxicity of certain oxidation species such as unsaturated fatty acids producing HETES etc has been thoroughly discussed (5). There is evidence that bilirubin is a biologic antioxidant (5, 6-9, 10). The oxidation of bilirubin has largely focused on the degradation of bilirubin between the pyrroles (11). Unfortunately there has been relatively little discussion concerning putative biological activity of the products of bilirubin oxidation (12). We recently reported on a new family of bilirubin oxidation products found following subarachnoid hemorrhage (13, 14, 15, 16). It appears that bilirubin oxidation products (BOXes) can be formed by the direct, non-enzymatic oxidation of bilirubin with hydrogen peroxide (14). In Figure 1 we see the relationship between bilirubin and BOXes (Adapted from Kranc et al (14)). Cleavage of bilirubin at the pyrrole, rather than between the pyrrole rings produces an apparently reactive mono-pyrrole amide (14). However, to date there has been relatively little
SAH and bilirubin oxidation

Figure 1. In this figure we see the relationship between bilirubin, BOX A and MVM. When bilirubin is oxidized between the pyroles the result is MVM. However, when bilirubin is oxidized by breaking the number 2 or 3 pyrrole, the result is BOXes and BOX A is represented here. BOX B is produced when the methyl and vinyl positions are switched, and occurs at the opposite end of the bilirubin.

information concerning the stability or characteristics of BOXes, which is important in understanding the role BOXes may play in subarachnoid hemorrhage induced vasospasm. Here we present our latest findings concerning the chemical characteristics of BOXes produced by the oxidation of bilirubin. The relatively unique characteristics of BOXes and their non-specific, non-enzymatic production provides important insight into the putative role of unconjugated bilirubin, and its oxidation products, in human pathology.

2.1. Aneurysmal subarachnoid hemorrhage

Subarachnoid hemorrhage from a ruptured saccular aneurysm continues to be a catastrophic neurological event that causes death or disability in a substantial percentage of patients. In those patients who receive medical care, the two major threats to recovery are rebleeding from the aneurysm and cerebral vasospasm induced by blood in the subarachnoid spaces. The use of early surgery or early coiling to mitigate the aneurysm has substantially decreased the threat of rebleeding. Unfortunately there is little progress in the prevention and treatment of vasospasm that continues to affect about 30-40% of patients and can lead to ischemic stroke. In the past two decades imaging methods and transcranial Doppler have been used to predict and monitor the onset of SAH-induced vasospasm. Angiography, however, remains the gold standard in the diagnosis of cerebral vasospasm. Transcranial Doppler is safe and essentially non-invasive, but its success and results can be operator dependent.

2.1.1. Cerebral vasospasm

Though many patients survive SAH, as many as 30-40% of the survivors suffer the complication of cerebral vasospasm following the initial hemorrhage, and a significant number of the patients with vasospasm have poorer outcomes. The delayed neurological deficits that occur following vasospasm are attributable to pathological vasoconstriction of vessels with secondary ischemia. We have proposed that the constriction is caused in part by an inability of the vascular smooth muscle to relax to normal physiological signals (17, 18).

The current pharmacological treatment of cerebral vasospasm following SAH uses a fairly cerebral arterial-specific voltage gated calcium channel antagonist, Nimodipine. Although calcium antagonists decrease the percentage of patients that have delayed neurological deficit following SAH, the drugs do not actually reverse the vasospasm. The failure of the vasospasm to respond to this therapy is consistent with the suggestion that smooth muscle contractile function could be the primary disturbance in vasospasm due to SAH.

2.1.2. Novel causes of vasospasm

A number of studies have addressed the possible mechanisms of vasospasm following SAH. However, the cause or causes have remained elusive. A leading candidate molecule for producing vasospasm has been the hemoglobin released from red blood cells following SAH. In figure 2 we present a scheme for the sequence of events thought to occur following SAH. Hemoglobin is postulated to act via scavenging of nitric oxide, a known vasodilator, but this action is short-lived. However, even though the occurrence of vasospasm correlates clinically with the amount of blood around vessels seen on CT scan, hemoglobin alone does not explain why vasospasm is commonly delayed 4-14 days following SAH. Infusions of pure hemoglobin into the subarachnoid space of rats do not produce brain injury/ischemic infarction, whereas infusions of lysed blood produce multi-focal areas of injury as demonstrated by stress protein induction. In addition, several laboratories have evidence that a small molecule present in CSF of SAH patients causes constriction of arteries. Thus, hemoglobin is likely not the sole cause of cerebral vasospasm.

We have searched for small molecules that could cause vasospasm of cerebral vessels, and have preliminary evidence that oxidized end-products of hemoglobin metabolism can constrict cerebral vessels in vitro and in vivo (presented below). Following SAH and release of hemoglobin from red blood cells, the heme is metabolized to biliverdin, CO and iron by heme oxygenase-2 found in blood vessels and meninges, and by heme oxygenase-1 that is induced in brain microglia. The biliverdin is metabolized to bilirubin, which causes the yellowish color seen in CSF following SAH. Bilirubin first appears around 12h following SAH, but does not peak until 3 or more days following SAH. Hence, the time course for the appearance of bilirubin is delayed following SAH, and if oxidized forms of bilirubin (BOXes, Bilirubin OXidized) play a role in vasospasm, this may help explain the time course of delayed vasospasm.

Though biliverdin and bilirubin are potent antioxidants, it was not known whether oxidized fragments of these molecules might exist and, it was not known if these compounds might cause vasoconstriction. Ischemia-
SAH and bilirubin oxidation

Figure 2. In this figure we see the putative sequence where subarachnoid hemorrhage leads to the degradation of the extravascular blood and eventual production of BOXes. Implicit in this scenario is the assumption that bilirubin is produced in quantities sufficient to produce BOXes and that there exists an environment consistent with the oxidation of bilirubin to BOXes.

Figure 3. This figure shows the concentration of hemeoxygnase I (HO-1) present in the CSF of SAH patients post SAH and the relationship to those patients with and without vasospasm. We used injured rat brain to act as a positive control for this experiment. The result is that CSF from patients with vasospasm (CSFv) has significantly more HO-1 compared to nonvasospastic CSF (CSFc). Control CSF is obtained from patients without SAH who have CSF drained for therapeutic reasons such as obstructive hydrocephalus.

reperfusion is known to produce reactive oxygen species and lipid peroxidation. Following SAH, marked increases in reactive oxygen species might occur because of the presence of extracellular heme, release of iron following metabolism of heme, and the influx of inflammatory cells into the CSF (Figure 2). In spite of this oxidative stress, little is known about the oxidation of other molecules such as bilirubin and heme. Our previous studies suggest that reactive oxygen species, particularly those from peroxides, produce oxidation products of bilirubin that are significantly vasoactive (14, 15, 16).

2.1.3. Hemorrhage

Immediately following a rupture of an aneurysm, leading to subarachnoid hemorrhage, arterial blood is injected into the subarachnoid space. The arterial blood mixes with the cerebral spinal fluid and comes in contact with the subarachnoid membranes. These membranes contain multiple enzymes including hemeoxygnase. This complex mixture of cells and enzymes is relatively poorly understood, but for approximately 50% of the SAH patients this mixture produces toxic vasoactive compounds. The events that occur following the hemorrhage include coagulation, hemolysis, oxidation, and degradation of cellular debris. The high concentration of oxygen, activation of HO-1, and activation of lymphocytes is likely to lead to bilirubin liberation and eventually to BOXes production. In Figure 3 we see that hemeoxygnase is elevated in the CSF of SAH patients with vasospasm. This is consistent with the thesis that increased bilirubin is needed for BOXes production in the vasospasm patient.

2.2. BOXes

2.2.1. Photostability of BOXes

These data are shown in Table 1. The resultant t½ for the degradation of BOXes is approximately 10±2 hours exposed to ambient indoor light (N=5). At 65°C, pH 7.5 in the dark, no decomposition was observed over the time course analyzed. We exposed the BOXes to UV light for 24 hours and found that there was essentially complete degradation of BOXes independent of concentration (Table 1). The kinetics observed were non-linear and involved a fast initial t½, sometimes as short as 1 hour, as well as a slower degradation component with an apparent t½ of about 7 hours. Overall t½ is as reported in Table 1. The BOXes are photosensitive as seen in Table 1.

Because BOXes are quickly degraded by light (ambient and sun), it may have made their characterization in humans rather difficult. It is relatively well known that the spinal fluid of the subarachnoid hemorrhage patients was vasoactive and cytotoxic (17, 19, 20), but only recently were the BOXes reported in the hemorrhage patients’ spinal fluid (39). Interestingly increased bilirubin in the spinal fluid of the subarachnoid hemorrhage patient has a time course that is similar to the reported incidence of vasospasm (21, 22).

The ability to generate BOXes from non-enzymatic oxidation, and the photodegradation of BOXes may have made them a difficult species to detect and understand. It is extremely likely that any time there is bleeding inside the body, third space blood, that the bilirubin being produced could be oxidized by the macrophages and similar cells that are activated under such conditions. Inside the body, there might be relatively little opportunity for photodegradation and the BOXes have potent biological effects. However, when the samples are removed for analysis the resulting exposure to light might result in photooxidation of BOXes and therefore, result in their apparent difficulty in being detected.

There is an extensive literature on physiological oxidations via reactive oxygen species such as H2O2 or oxygen radicals like O2•- (23, 24, 25, 26, 27, 28, 29, 30, 31). Lipid and protein oxidations in biological systems have also been well studied (3, 32, 33, 34, 35, 36). More recently bilirubin oxidation has started to gain interest and attention.
Reactions with O$_2$ or related reactive oxygen species can cause free oxygen to be released from oxyhemoglobin because free oxygen can be maintained at high levels as in the arterial blood following subarachnoid hemorrhage. Peroxides, OH$^-$, and O$_2$ can occur because of oxygen accepting free electrons and or neurotoxic. This includes HETES, lipid peroxides, epoxides, nitrosylated tyrosine, MDA and bilirubin oxidation products. Thus any one or a combination therein may be a marker for if not contributor to subarachnoid hemorrhage induced vasospasm.

<table>
<thead>
<tr>
<th>Table 1. Photostability of BOXes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Condition</strong></td>
</tr>
<tr>
<td>Direct sunlight, 25°C, PBS, pH 7.5</td>
</tr>
<tr>
<td>Ambient light, 25°C, PBS, pH 7.5</td>
</tr>
<tr>
<td>Ambient light, 40°C, PBS, pH 7.5</td>
</tr>
<tr>
<td>Ambient light, 40°C, PBS, pH 6</td>
</tr>
</tbody>
</table>

No light, 65°C, PBS, pH 7.5         None detected within exp. error, n=5.

Spectrophotometer cell, Visible Lamp, 330-550 nm, 25°C, PBS, pH 7.5 None detected within exp. error, n=5.

Abbreviations: PBS= Phosphate buffered saline.

What is lacking, however, is an understanding of the resultant species and biological importance of the compounds made post oxidation of bilirubin (13, 15, 16, 41).

2.2.2. BOXes production

There are reports of differences between the reactivity and chemical characteristics of the geometrical and structural isomers of bilirubin (42). It is unknown whether these differences affect BOXes production. However, to determine the pathway for BOXes production one may need to understand the isomers involved in the reactions (14). In biological systems the inter-conversion of the EE, EZ, ZZ and cyclic forms is known to occur (42). The resultant isomers of BOXes (BOX A and BOX B) are not necessarily due to different isomers of bilirubin (the EE, EZ and ZZ forms), but rather due to oxidation of either ‘end’ of the bilirubin molecule yielding the alternate positions of methyl and vinyl. BOXes’ apparent chemical uniqueness to come from the amide group derived from the bilirubin’s cleavage at the 2 or 3 pyrrole.

2.2.3. Oxidative stress

Oxidative stress following subarachnoid hemorrhage is well characterized. The oxidative stress can occur because of oxygen accepting free electrons coming from hemoglobin following the hemorrhage. The white blood cells, arachnoid cells, and infiltrating inflammatory cells all can be present in or near the hemorrhage and contribute to the production of reactive oxygen species. Reactive oxygen species as well as oxygen free radicals include; O$_2$$^-$, H$_2$O$_2$, NO, lipid peroxides, OH$,^-$ and O$_2$. The role of diatomic oxygen (O$_2$) as a reactive oxygen species may be very important in the arterial blood following subarachnoid hemorrhage because free oxygen can be maintained at high levels as the bound oxygen is liberated from oxyhemoglobin.

Reactions with O$_2$ or related reactive oxygen species can be facilitated by several metal ions. The above listed compounds can form covalent bonds with numerous molecules found in CSF. The result is covalently modified compounds that may be vasoactive, vasotoxic and or neurotoxic. This includes HETES, lipid peroxides, epoxides, nitrosylated tyrosine, MDA and bilirubin oxidation products. Thus any one or a combination therein may be a marker for if not contributor to subarachnoid hemorrhage induced vasospasm.

2.2.4. Hemorrhage

Hemorrhagic complications and the mechanisms for such complications have been an anathema and enigma in medical research. Residual dysfunction following hemosiderin staining is a common observation, but the molecule or molecules involved are unknown (43, 44). Hyperbilirubinemia or kernicterus can affect infants and cause profound neurological disturbances. The treatment is phototherapy and assumed to be beneficial by clearing the bilirubin (45). However, we posit that the hyperbilirubinemic patient may benefit from photodegradation of BOXes. Because BOXes have only been found in clinical conditions in low concentrations (16) and because they are photolabile (14) it may have been difficult to characterize and understand their role in certain diseases. It is important now to better understand the chemistry of BOXes and determine what if any role they play in biology and medicine.

BOXes have been found in the micromolar range in human spinal fluid following subarachnoid hemorrhage (16) and their concentration is associated with cerebral vasospasm (16). Bilirubin levels rise in the spinal fluid following subarachnoid hemorrhage (46) and their time course correlates well with the accepted time course of cerebral vasospasm, but bilirubin is not vasoactive. Thus there has been interest in bilirubin’s association with vasospasm with mixed results (13, 16, 21, 22, 47, , 48, 49, 50, 51, 52, ). A bilirubin derivative such as BOXes is therefore quite consistent with the clinical characteristics of vasospasm. In vitro and in vivo studies suggest that they may be involved in subarachnoid hemorrhage induced cerebral vasospasm (13, 15, 16, 47,). In the brain little light would pass through the skull and as such BOXes might accumulate and contribute to the pathology of subarachnoid hemorrhage induced cerebral vasospasm.

2.2.5. BOXes and biochemical oxidations

There has been interest concerning the source of bilirubin to form BOXes, because this step in the formation of BOXes offers promise as a therapeutic target. BOXes can be produced by oxidizing bilirubin with hydrogen peroxide in vitro. However, it is not entirely clear if reactive oxygen species (ROS), such as hydrogen peroxide, are the primary oxidizers of bilirubin in vivo. Recently, our group studied potential oxidizers of bilirubin in an in vitro model of SAH (53).

In order to examine the oxidation of bilirubin by oxidases, we used a system that would mimic physiological conditions encountered during the oxidative stress of stroke. To this system was added substances determined to inhibit or accelerate the production of BOXes. Such substances were determined to be mononucleuc leucocytes and other blood cells, human blood plasma, cerebral spinal fluid, peroxide, catalase, cytochrome C, superoxide dismutase, heme oxygenase, molecular oxygen, cyanide, absence of molecular oxygen (nitrogen gas atmosphere), mechanical cell damage and ascorbate. The incubations were performed in sealed tubes for the experiments that included air displacement with volatile gases. The incubation times were 72 hours at 37 degrees centigrade with mild agitation. BOXes production was monitored by

(8, 9, 10, 16, 37-40, ).
SAH and bilirubin oxidation

Table 2. BOXes production

<table>
<thead>
<tr>
<th>Description of Incubation conditions</th>
<th>% BOXes production (different from control)</th>
<th>n</th>
<th>Incubation condition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>16±3</td>
<td>16</td>
<td>A</td>
</tr>
<tr>
<td>MNLs</td>
<td>33±4</td>
<td>6</td>
<td>B</td>
</tr>
<tr>
<td>CN</td>
<td>19±6</td>
<td>10</td>
<td>C</td>
</tr>
<tr>
<td>Peroxide</td>
<td>22±3</td>
<td>10</td>
<td>D</td>
</tr>
<tr>
<td>Lysis</td>
<td>4±6</td>
<td>8</td>
<td>E</td>
</tr>
<tr>
<td>Lysis/Ascorbate</td>
<td>329±60</td>
<td>10</td>
<td>F</td>
</tr>
<tr>
<td>Cytidine/Catalase+SOD</td>
<td>5±7</td>
<td>10</td>
<td>G</td>
</tr>
<tr>
<td>MNLs/Catalase+SOD</td>
<td>83±47</td>
<td>8</td>
<td>H</td>
</tr>
<tr>
<td>Nitrogen Gas</td>
<td>22±8</td>
<td>10</td>
<td>I</td>
</tr>
<tr>
<td>Catalase</td>
<td>34±15</td>
<td>10</td>
<td>J</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>62±6</td>
<td>6</td>
<td>K</td>
</tr>
<tr>
<td>Cytidine/Ascorbate</td>
<td>68±8</td>
<td>10</td>
<td>L</td>
</tr>
<tr>
<td>Cytidine/Lysis/Ascorbate</td>
<td>74±9</td>
<td>10</td>
<td>M</td>
</tr>
<tr>
<td>Lysis/Cytidine</td>
<td>69±14</td>
<td>10</td>
<td>N</td>
</tr>
<tr>
<td>Oxygen Gas</td>
<td>55±25</td>
<td>10</td>
<td>O</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>45±6</td>
<td>10</td>
<td>P</td>
</tr>
<tr>
<td>Control</td>
<td>0±2</td>
<td>10</td>
<td>Q</td>
</tr>
</tbody>
</table>

A. Incubation contained blood with intact cells, CSF, and 1.75 mM HO-1. Reactions were incubated for 72 hrs., B. Incubation A including MNLs, C. Incubation A including 3.2 mM cyanide, D. Incubation A including 13 mM hydrogen peroxide, E. Incubation A including cells lysed by sonication, F. Incubation E including 1.3 mM ascorbate, G. Incubation A including 1920 U/mL catalase and 50 U/mL SOD and 3.2 mM cyanide, H. Incubation B including 1920 U/mL catalase and 50 U/mL SOD, I. Incubation A after nitrogen gas bubbled for 5 minutes through the solution, J. Incubation A including 13 mM ascorbate added, L. Incubation A including 3.2 mM cyanide and 1.3 mM ascorbate, M. Incubation E including 3.2 mM cyanide and 1.3 mM ascorbate, N. Incubation B including 3.2 mM cyanide added, O. Incubation A after oxygen bubbled through the solution for 5 minutes, P. Incubation A including 10 micromolar cytochrome C added, Q. Incubation A without 1.75 mM HO-1.

Abbreviations: CSF, Cerebrospinal Fluid; MNL, mononuclear leukocytes; SOD, Superoxide dismutase.

![Figure 4](image.png)

Figure 4. This figure shows the apparent rates of BOXes production in vitro and varying pH. The enzyme used for this oxidation was horseradish peroxidase. We see that the optimum pH for the production of BOXes from bilirubin is at or near physiological pH of 7.0.

standard methods developed in our laboratory using optical spectroscopy and HPLC. The data obtained in Table 2 is a compilation of published and unpublished data with the resultant experimental conditions (N≥5). As the results indicate, sometimes the mixture of one or two added substances provided significant inhibition or acceleration of the production of BOXes. This indicates that the biochemical production of BOXes occurs by a complex process that undoubtedly involves cytochrome oxidase. The individual oxidative species that might be involved in this process are at this time not clearly understood.

From these data it appears that oxygen and/or cytochrome oxidase may be more important for BOXes production than ROS. A number of results support this hypothesis. First oxygen is needed in the reaction, while adding H2O2 at physiological amounts did not increase BOXes. Mononuclear leukocytes (MNLs) did not increase BOXes, however, adding the antioxidant enzymes catalase and superoxide dismutase to MNLs paradoxically increased BOXes compared to control. This could be explained by the fact that both enzymes produce molecular oxygen as a product. The oxygen then stimulates cytochrome oxidase. Also, the production of BOXes post cell lysis and addition of ascorbate is greatly increased. We believe that the cell lysis makes bilirubin more accessible to cytochrome oxidase and ascorbate stimulates the enzyme by transferring electrons to it. The role of enzymatic production of BOXes in vivo is unknown at this time, but we have found that enzymatic production in vitro is quite feasible and follows standard enzymatic methods. In Figure 4 we see the pH dependence of BOXes production with horseradish peroxidase. Finally, stimulating cytochrome oxidase by adding cytochrome c also increased the production of BOXes. Important findings from this study are summarized in Table 2.

These data suggest a central role for oxygen and/or cytochrome oxidase in making BOXes in this in vitro model and by inference after SAH. Interestingly Hansen et al (37, 38, 40) suggested that cytochrome oxidase may be involved in oxidizing bilirubin in neonatal jaundice. However, they did not assay for BOXes production but rather a decrement in bilirubin. Cytochrome oxidase is likely to be only one of several important mechanisms by which bilirubin is oxidized to BOXes following SAH.

3. CONCLUSION

We have discussed some of the chemical events that occur in the hemorrhagic milieu following subarachnoid hemorrhage and which may be involved in the pathogenesis of cerebral vasospasm. The oxidation of bilirubin to produce BOXes is associated with vasospasm and BOXes are vasoactive in vitro. Notwithstanding the chemical environment needed to oxidize bilirubin to BOXes is most definitely present in the SAH patient. We therefore conclude that bilirubin oxidation may be associated with the pathological sequelae associated with vasospasm following SAH.

4. ACKNOWLEDGEMENTS

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**Key Words:** Bilirubin, Oxidative Stress, Subarachnoid Hemorrhage, Aneurysmal Stroke

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