Intracisternal Increase of Superoxide Anion Production in a Canine Subarachnoid Hemorrhage Model

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Background and Purpose—Reactive oxygen species (ROS) are thought to be primary in the pathogenesis of cerebral vasospasm after subarachnoid hemorrhage (SAH). However, as direct evidence of ROS has not yet been demonstrated in cerebral vasospasm, we sought to substantiate superoxide anion (\(O_2^−\)) generation in the subarachnoid space after SAH using a modification of Karnovsky’s manganese/diaminobenzidine (Mn\(^{2+}/DAB\)) technique.

Methods—SAH or sham operation was induced according to a 2-hemorrhage model in a total of 24 beagle dogs. On day 2 or 7 after SAH or sham operation, dogs were intrathecally infused with buffer containing Mn\(^{2+}\) and DAB, and the brain stem was prepared for light and electron microscopy. Possible colocalization of ferrous (Fe\(^{2+}\)) or ferric (Fe\(^{3+}\)) iron ions with \(O_2^−\) was also examined with the use of Turnbull blue or Berlin blue staining, respectively.

Results—Light microscopy revealed amorphous, amber deposits within the subarachnoid hematoma, the periarterial space, and the tunica adventitia of the basilar artery on days 2 and 7 after SAH. \(O_2^−\) deposits were eliminated by addition of superoxide dismutase or exclusion of either Mn\(^{2+}\) or DAB from the perfusate, confirming the specificity of the reaction. These deposits were colocalized with blue reaction deposits indicating Fe\(^{2+}\) and Fe\(^{3+}\). Within the subarachnoid space, \(O_2^−\) indicating electron-dense fine granules were preferentially located around degenerated erythrocytes and, secondarily, infiltrating macrophages and neutrophils.

Conclusions—We show direct evidence for enhanced production of \(O_2^−\) and Fe\(^{2+}/Fe^{3+}\) iron ions in the subarachnoid space after SAH, lending further support to the pathogenic role of ROS in cerebral vasospasm after SAH. (Stroke. 2001;32:636-642.)

Key Words: reactive oxygen species ■ subarachnoid hemorrhage ■ superoxides ■ vasospasm, intracranial ■ dogs

Lipid peroxidation and other consequences of increased levels of reactive oxygen species (ROS) have been implicated in the etiology of cerebral vasospasm after subarachnoid hemorrhage (SAH).\(^1\)–\(^3\) It is generally thought that the primary contributor to ROS production after SAH is the autoxidation within the subarachnoid space of oxyhemoglobin (oxyHb) to met-Hb.\(^4\) As a direct product of this redox reaction, superoxide anion (\(O_2^−\)) is converted to highly reactive hydroxyl radical (\(\cdot OH\)) through the metal-catalyzed Haber-Weiss and Fenton reactions.\(^5\)–\(^6\) In support of the theory that ROS are primary pathogens for SAH, a variety of antioxidants have been shown to attenuate cerebral vasospasm in animals and humans.\(^7\)–\(^14\) We have shown that intracisternal overproduction of \(O_2^−\) may initiate and/or mediate cerebral arterial vasoconstriction and subsequent structural damage.\(^3\) Moreover, administration of ferrous (Fe\(^{2+}\)) or ferric (Fe\(^{3+}\)) iron chelators was shown to mitigate against cerebral vasospasm, providing evidence that the iron-catalyzed Haber-Weiss and Fenton reactions are involved in the mechanism of ROS generation leading to the occurrence of cerebral vasospasm.\(^15\)–\(^17\)

Evidence for ROS production in vivo after SAH has been assessed by indirect approaches such as measurement of lipid peroxide production and quantification of superoxide dismutase (SOD) and glutathione peroxidase activities.\(^18\) Although more direct approaches have been used for the detection of ROS in brain tissue, such as nitroblue tetrazo-lium,\(^19\) electron spin resonance,\(^20\) fluorochrome sensor,\(^21\) and histochemical methods,\(^22\) direct detection in situ of ROS, including \(O_2^−\), has not yet been performed for cerebral vasospasm after SAH.

In this study we used a modification of Karnovsky’s manganese/diaminobenzidine (Mn\(^{2+}/DAB\)) technique that we have previously reported.\(^22\) This method yields an amber osmiophilic polymer that is formed in the presence of \(O_2^−\), Mn\(^{2+}\), and DAB and can be visualized under both light and electron microscopes.\(^23\) Using this technique, we sought to elucidate in this study the major sites and cellular species...
responsible for $\cdot{\mathrm{O}}_{2}^{-}$ production in the subarachnoid space after SAH. As the Haber-Weiss and Fenton reactions require iron ions as cofactors to yield other species of ROS, we also investigated whether Fe$^{2+}$ or Fe$^{3+}$ iron ions coexisted with $\cdot{\mathrm{O}}_{2}^{-}$, using Turnbull blue or Berlin blue staining, respectively.

**Materials and Methods**

**Reagents and Buffer Compositions**

Reagent-grade MnCl$_2$, C$_6$H$_5$Na$_3$O$_7$, CaCl$_2$, KCl, NaCl, Na$_2$O, NaOH, 0.2 mol/L phosphate buffer solution (pH 7.4), Tris, and 3.3'-DAB were obtained from Sigma. Glutaraldehyde and paraformaldehyde were purchased from TAAB Laboratories Equipment. Buffer (solution A, adjusted to pH 7.4 by titration with 0.5N NaOH and then filtered) contained MnCl$_2$ (40 mmol/L), C$_6$H$_5$Na$_3$O$_7$ (40 mmol/L), CaCl$_2$ (2 mmol/L), KCl (4 mmol/L), Tris (4 mmol/L), and Na$_2$O (1 mmol/L), as previously reported.$^9$ The DAB stock solution was made by dissolving DAB in distilled water (10 mg DAB per milliliter). To prevent DAB precipitation, DAB stock solution was made by slowly adjusting the pH to 5.0 with the drop-by-drop addition of 0.5N NaOH (approximately 3 mL/100 mL) during vigorous stirring (solution B). The final Mn$^{2+}$/DAB buffer containing 2.5 mmol/L DAB was prepared immediately before use by mixing solutions A and B together in a 9:1 ratio. To confirm that positive reaction indicated the presence of $\cdot{\mathrm{O}}_{2}^{-}$, a Mn$^{2+}$/DAB buffer containing human recombinant Cu/Zn superoxide dismutase (hrSOD) and separate buffers containing no Mn$^{2+}$ (non-Mn$^{2+}$) or no DAB (non-DAB) were prepared. HrSOD (kindly provided by Nippon Kayaku Co, Tokyo, Japan; lot No. 10125S) was dissolved immediately before use in the Mn$^{2+}$/DAB buffer to a final concentration of 5×10$^{-3}$ M/mL. In the non-Mn$^{2+}$ buffer, 80 mmol/L NaCl replaced the 40 mmol/L MnCl$_2$. In the non-DAB buffer, an equal amount of distilled water replaced the DAB stock solution.

**Surgical Procedures**

Animal housing and care and the present protocols complied with the Principles of Laboratory Animal Care and the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services publication No. [NIH] 85-23, revised 1985) and have previously been approved by the Animal Use Ethical Committee of the Saitama Medical School.

**Subarachnoid Hemorrhage Model**

Twenty-four adult beagle dogs of either sex, weighing 10 to 15 kg, were randomly assigned to treatment groups. Animals were anesthe-
tized with injection of sodium pentobarbital (30 mg/kg IV), orally intubated, and fixed in a stereotaxic device. Animals were main-
tained by mechanical ventilation with room air throughout the experiment. To rule out fluctuation of diameter of the cerebral artery during the course of the experiment. The cisternal injection of arterial blood was repeated 48 hours later by the same method. Additional angiography was performed to confirm the narrowing of the basilar artery on day 2 or 7. In the animals subjected to sham treatment, an identical amount of physiological saline was injected into the cisterna magna, and angiography was performed on days 2 and 7 according to the method described below.

**Evaluation of the Basilar Artery Diameter**

The diameter of the basilar artery was measured at 5 predetermined locations along the vessel on the angiogram with an optical microm-
eter. Examination of all films was performed in a blind fashion by a single investigator. Arterial narrowing on the angiogram was ex-
pressed as a percentage of the basilar artery diameter of the baseline angiogram obtained on day 0 in the same animals, and the accumu-
lated angiography data in SAH and sham groups were expressed as mean±SE. For statistical comparisons, ANOVA was used. A $P$ value of <0.05 was considered statistically significant.

**Intrathecal Perfusion Procedure**

After angiography on day 2 or 7, a 4-cm-long midline skin incision was made from the external occipital protuberance was made under general anesthesia. The muscular muscles were divided in the midline, exposing the atlanto-occipital membrane. Through a small incision made in the membrane, 2 silicon catheters (1.2 mm in diameter, 10 cm long) were separately inserted into either side of the cerebel-
lomedullary cisterns. One was connected to the perfusion tube, while the other was connected to the draining tube. The length of the catheters in the subarachnoid space was approximately 1.5 cm, which was sufficient to reach the preponinte cistern. The opening of the atlanto-occipital membrane was closed in a watertight fashion by the use of a surgical adhesive material. Then the basal cistern was perfused for 10 minutes with 10 mL Mn$^{2+}$/DAB buffer on day 2 (group 1; n=6) or 7 (group 2; n=6). Separate groups of animals were similarly perfused on day 7 with Mn$^{2+}$/DAB buffer containing hrSOD (group 3; n=3), non-Mn$^{2+}$ (group 4; n=3), or non-DAB buffer (group 5; n=3). Animals subjected to sham treatment were perfused with Mn$^{2+}$/DAB buffer on day 7 (group 6; n=3). The preliminary study, in which 3 dogs were subjected to perfusion using identical amounts of dye (4% toluidine blue–saline) solution, had shown that the basal as well as the preponinte cisterns were satisfactorily perfused by the above technique.

**Pathological Studies**

After intrathecal perfusion with the designated perfusate, animals were euthanatized by an injection of sodium pentobarbital (50 mg/kg IV) followed by exsanguination. The basilar artery together with the brain stem was immediately harvested and processed for light and electron microscopy. Sections for light microscopy were immersed in a phosphate buffer containing 4% paraformaldehyde, routinely embedded in paraffin, sectioned (3 m), counterstained with 4% toluidine blue or Berlin blue staining, by 1% osmium tetroxide fixation, dehydrated through a graded series of ethanol solutions, transferred to propylene oxide, and embedded in Quetol 812. Serial semithin sections (1 m) from resin-embedded blocks were then stained with 1% safranine/0.5% toluidine blue. Ultrathin sections (70 nm) were double-stained with uranium acetate and lead citrate and were examined at 75 kV with a Hitachi H-7000 transmission electron microscope.

**Evaluation of $\cdot{\mathrm{O}}_{2}^{-}$ and Fe$^{2+}$/Fe$^{3+}$ Reaction Products**

Using light microscopy, amounts of $\cdot{\mathrm{O}}_{2}^{-}$ and Fe$^{2+}$/Fe$^{3+}$ reaction products were graded in a semiquantitative fashion as absent (−), slight (+), mild (+), or moderate (+++) in the subarachnoid space as well as the basilar arterial wall in a blinded fashion. Sections graded as absent or slight were further examined by electron microscopy. Statistical analysis was performed by the Kruskal-Wallis test followed by post hoc comparison by the Mann-Whitney U test. A $P$ value of <0.05 was considered statistically significant.
-\(\text{O}_2^-\) and Fe\(^{2+}/\text{Fe}^{3+}\) Iron Reaction Deposits After SAH

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of Animals</th>
<th>Experimental Condition</th>
<th>Perfusion Solution</th>
<th>Subarachnoid Space</th>
<th>Tunica Adventitia of Basilar Artery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>SAH (day 2)</td>
<td>Mn(^{2+}/\text{DAB})</td>
<td>0/0 0/0 2/2 4/4</td>
<td>0/0 2/2 4/4 0/0</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>SAH (day 7)</td>
<td>Mn(^{2+}/\text{DAB})</td>
<td>0/0 0/0 0/0 6/6</td>
<td>0/0 0/0 6/6 0/0</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>SAH (day 7)</td>
<td>Mn(^{2+}/\text{DAB}) with hrSOD</td>
<td>*3/0 0/0 0/0 0/3</td>
<td>*3/0 0/0 0/3 0/0</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>SAH (day 7)</td>
<td>Non-Mn(^{2+})</td>
<td>*3/0 0/0 0/0 0/3</td>
<td>*3/0 0/0 0/3 0/0</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>SAH (day 7)</td>
<td>Non-DAB</td>
<td>*3/0 0/0 0/0 0/3</td>
<td>*3/0 0/0 0/3 0/0</td>
</tr>
<tr>
<td>6</td>
<td>3</td>
<td>Sham (day 7)</td>
<td>Mn(^{2+})</td>
<td>0/3 *3/0 0/0 0/0</td>
<td><em>3/3</em> 0/0 0/0 0/0</td>
</tr>
</tbody>
</table>

Reaction deposit amounts were graded as absent (−), slight (+), mild (++), or moderate (+++).

Data are represented as -\(\text{O}_2^-/\text{Fe}^{2+}\) and \(\text{Fe}^{3+}\) reaction deposits since differences were not apparent when comparing -\(\text{O}_2^-\) reaction deposits colocalized with \(\text{Fe}^{2+}\) vs those found with \(\text{Fe}^{3+}\) deposits.

*Significant \((P<0.05)\) differences comparing each experimental group with group 1 or 2.

Results

Evaluation of the SAH Model and Measurement of Basilar Artery Diameter

During the course of the study, no animals died, and no focal neurological deficits developed. On postmortem examination of animals subjected to SAH, the basilar artery was embedded in the clot of injected autologous blood that encased the surrounding cisterns. None of the animals subjected to sham treatment had SAH in the basal cistern.

In animals subjected to sham treatment, there was no significant time-dependent change in the basilar artery diameter (99.3±2.4% \([\text{mean}\pm\text{SE}]\) on day 2 and 98.5±2.1% on day 7; \(n=3\) for group 6; \(P>0.05\)). A statistically significant difference in the basilar artery diameter was revealed between the SAH groups and the sham treatment group \((P<0.01\) for each comparison) on either day 2 or 7. In animals subjected to SAH, the basilar artery diameter was reduced to 72.1±2.8% of the control on day 2 (data assembled from groups 1 to 5; \(n=21\)). The reduction in basilar artery diameter on day 7 (58.6±3.2%, data assembled from groups 2 to 5; \(n=15\)) was significantly greater than that on day 2 \((P<0.05)\).

Histochemical Distributions of Reaction Products of -\(\text{O}_2^-\), \(\text{Fe}^{2+}\), and \(\text{Fe}^{3+}\)

Dotlike or speckled amber reaction deposits were visible even by surgical microscopy in groups 1 and 2 (Mn\(^{2+}/\text{DAB}\) buffer) at the time of death on days 2 and 7 after SAH. Interestingly, small amounts of dotlike amber reaction products were observed on the dorsal and ventral surfaces of the brain stem in group 6 (sham treatment). Reaction deposits were not observed on the dorsal and ventral surfaces of the brain stem in animals subjected to SAH. In groups 3 and 4 (perfused with Mn\(^{2+}/\text{DAB}\) buffer containing hrSOD, 4 (non-Mn\(^{2+}\)), and 5 (non-DAB), no reaction deposits of -\(\text{O}_2^-\) were observed in either the subarachnoid space or the basilar arterial wall (Figure 1). In groups 1 to 5, Turnbull blue or Berlin blue staining revealed the presence of a significant amount of amorphous blue reaction deposits, indicating the presence of \(\text{Fe}^{2+}\) or \(\text{Fe}^{3+}\) iron ions, around the basilar artery and degenerated erythrocytes in the subarachnoid space. It is important to note that these blue reaction deposits were found in the close vicinity of -\(\text{O}_2^-\) reaction deposits. Considerable amounts of reaction deposits were also observed within the tunica adventitia of the basilar arterial wall (Figure 2). While the aforementioned pattern of colocalization of -\(\text{O}_2^-\) reaction deposits and \(\text{Fe}^{2+}\) or \(\text{Fe}^{3+}\) reaction deposits within the subarachnoid space and the basilar arterial wall was similar between days 2 and 7, the amount of those deposits was slightly, but not significantly, increased on day 7.

In group 6, no \(\text{Fe}^{2+}\) or \(\text{Fe}^{3+}\) reaction products were observed in either the subarachnoid space or the basilar arterial wall.

Electron microscopic examination in groups 1 and 2 revealed that electron-dense fine granules of varying size indicating -\(\text{O}_2^-\) were abundantly present in the subarachnoid space, predominately located around degenerated erythrocytes and, much more secondarily, in the vicinity of infiltrating macrophages and neutrophils. Considerable numbers of granules were attached to the outer surface of the cytoplasmic membrane of those cells. In the basilar arterial wall, -\(\text{O}_2^-\) reaction deposits were also detected in the vicinity of infiltrating macrophages and neutrophils in the tunica adventitia of the basilar artery (Figure 3.). In group 6 on day 7, small amounts of -\(\text{O}_2^-\) reaction products were present in the subarachnoid space juxtaposed to the arachnoid membrane and the pia mater. No -\(\text{O}_2^-\) reaction deposits were identified by electron microscopy in groups 3, 4, or 5 in either the subarachnoid space or the basilar arterial wall.

In the basilar arterial wall of groups 1 to 5, mild to moderate pathological changes were observed in endothelial and smooth muscle cells on days 2 and 7. These changes include increased number of a variety of cytoplasmic organelles...
(mitochondria, lysosomes, and rough endoplasmic reticulum), cytoplasmic vacuoles containing fine granules, myelin figures or more amorphous material, and detached intracellular junctions. These pathological findings appeared more conspicuous with time. Furthermore, in groups 1 and 2, although moderate vacuole formation and destruction of collagen fibers in the tunica adventitia close to the deposits was observed, there were no necrotic cell changes in the tunica media and intima. In addition, small numbers of activated macrophages were observed in the tunica adventitia. In group 6, no abnormal findings were observed in any of the specimens.

**Discussion**

It is becoming increasingly substantiated that ROS and subsequent lipid peroxidation participate in the etiology of cerebral vasospasm after SAH. However, as direct evidence has been lacking regarding generation of ROS in the subarachnoid space after SAH, we wished to develop and characterize a direct method to visualize ROS, and in particular -O_2^-*, after SAH induction. Among a variety of methods to detect ROS, we chose a modification of Karnovsky's Mn^{2+}/DAB technique for the following reasons. First, the detection of -O_2^-* is of utmost importance because it is the initially generated species of ROS leading to subsequent generation of other ROS. Second, the aforementioned technique can be combined with other histochemical methods to examine the coexistence of Fe^{2+} and Fe^{3+} iron ions, which are critical cofactors for the Haber-Weiss and Fenton reactions, with -O_2^-*. Third, light and electron microscopic examination is expected to clarify the topographical distribution of -O_2^-* and iron ions within the subarachnoid space, thereby helping to identify the origin as well as the immediate target of -O_2^-*.

By the use of the aforementioned technique, we were able to confirm that SAH leads to generation of -O_2^-* as follows.

**Figure 1.** Coronal sections from dogs after SAH treatment. Panels A through C represent the basilar artery, and panels D through F show the subarachnoid space. On days 2 (A, D) and 7 (B, E) after SAH, amorphous, amber reaction deposits that indicated -O_2^-* production were seen surrounding infiltrating macrophages and neutrophils as well as extravasated erythrocytes in the subarachnoid space of dogs perfused with Mn^{2+}/DAB buffer (A, B, D, E). In contrast, no reaction deposits of -O_2^-* were observed in dogs perfused with Mn^{2+}/DAB buffer containing hrSOD buffer (C, F) on day 7 after SAH, indicating the -O_2^-* specificity of these reaction deposits. Each section was counterstained with 4% methyl green. Magnification ×100.
Light and electron microscopy revealed that \( \cdot \text{O}_2^- \) reaction deposits were primarily located around erythrocytes. However, a significant amount of deposits was also observed around infiltrating macrophages and neutrophils in the extracellular space of the subarachnoid hematoma, the periarterial space, and the tunica adventitia of the basilar artery on days 2 and 7 after SAH. Furthermore, Turnbull blue and Berlin blue staining revealed that these \( \cdot \text{O}_2^- \) reaction deposits were tightly colocalized with \( \text{Fe}^{2+} \) or \( \text{Fe}^{3+} \) reaction deposits, providing evidence that Haber-Weiss and Fenton reactions are taking place in the subarachnoid space after SAH.

**Confirmation of \( \cdot \text{O}_2^- \) Reaction Deposits**

The rationale of the histochemical technique for the detection of \( \cdot \text{O}_2^- \) production is as follows:

1. \( \cdot \text{O}_2^- + \text{Mn}^{2+} + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{Mn}^{3+} \)
   
   \( \text{K}_1=6 \times 10^6 \text{ mol/L}^{-1}\text{s}^{-1} \)

   \( \text{Mn}^{3+} + \text{DAB} \rightarrow \text{osmiophilic polymer}^{25} \)

2. \( \cdot \text{O}_2^- + \text{SOD} \rightarrow \frac{1}{2} \text{H}_2\text{O} + \frac{1}{2} \text{O}_2 + \text{SOD} \)

   \( \text{K}_2=10^9 \text{ mol/L}^{-1}\text{s}^{-1} \)

Reactions 1 and 2 are competitive; however, the rate constant (\( \text{K}_2 \)) of reaction 2 is 3 orders of magnitude greater than the rate constant (\( \text{K}_1 \)) of reaction 1. Thus, if reaction 1 is halted by the addition of a nonlimiting amount of SOD (experimental group 3), reaction deposits formed in the absence of SOD (experimental groups 1 and 2) could be interpreted as specific evidence of \( \cdot \text{O}_2^- \) production. Such a confirmatory strategy revealed that, in dogs subjected to SAH, \( \cdot \text{O}_2^- \) reaction deposits were completely diminished when an excess amount of hrSOD was added to the \( \text{Mn}^{2+}/\text{DAB} \) buffer (experimental group 3). As additional controls to determine the specificity of the reaction deposits produced by the complete reaction buffer, we eliminated DAB or \( \text{Mn}^{2+} \) from the perfusion buffer (experimental groups 4 and 5). As expected, no reaction deposits were present in these conditions. Additionally, sodium azide (1 mmol/L, a concentration at which the enzymatic activities of CuZn SOD or Mn SOD

**Figure 2.** Coronal sections stained with Turnbull blue from dogs after SAH treatment. These sections were stained with the ferrous ion (Fe\(^{3+}\))-specific Turnbull blue stain, and the basilar artery (A) and subarachnoid space (B) are shown. In both regions, \( \cdot \text{O}_2^- \) reaction deposits were detected together with amorphous, blue reaction deposits of Fe\(^{3+}\). Magnification ×100.

**Figure 3.** Electron micrograph shows the subarachnoid space on day 7 after SAH from a dog perfused with \( \text{Mn}^{2+}/\text{DAB} \) buffer. Numerous reaction deposits of varying size were present in the subarachnoid space. These electron-dense granules were detected mainly in the extracellular space, in close apposition to the cytoplasmic membranes of infiltrating macrophages and neutrophils. Bar=4 \( \mu \text{m} \).
are not inhibited) was added to all perfusates to block the nonspecific osmophilic polymerization between DAB and endogenous peroxidase, catalase, and mitochondrial cytochrome oxidase enzymes. Furthermore, to examine the possibility of nonspecific polymerization of DAB by endogenous H₂O₂, we added 3% H₂O₂ to the Mn²⁺/DAB buffer. The addition of H₂O₂ did not elicit the formation of amber reaction deposits. Taken together, our results indicate that the reaction deposits observed in experimental groups 1 and 2 are specifically due to enhanced production of -O₂⁻. Noteworthy is the finding that small numbers of -O₂⁻ reaction deposits were observed in the subarachnoid space abutting the arachnoid and pia mater in sham-treated animals subjected to perfusion with the Mn²⁺/DAB buffer (experimental group 6). The aforementioned result may be consonant with the basic physiological production of -O₂⁻ in the subarachnoid space, which may have been enhanced as a response to sham operation.

**Possible Origin of -O₂⁻ and Fe²⁺/Fe³⁺ Iron Ions**

Although oxyHb liberated into the subarachnoid space through clot lysis has been deemed to be the primary source of ROS and iron ions, this thesis has suffered from a lack of direct evidence. In this regard, the present study provides histochemical evidence for enhanced production of -O₂⁻ and its coexistence with Fe²⁺ or Fe³⁺ iron ions in the subarachnoid space on days 2 and 7 after SAH. While -O₂⁻ reaction deposits were detected within the subarachnoid hematoma, the periartrial space, and the tunica adventitia of the basilar artery, they were particularly abundant around degenerated erythrocytes and, secondarily, infiltrating macrophages and neutrophils. However, we did not detect deposits in the tunica media or intima. In this regard, it is important to note whether the Mn²⁺/DAB perfusate used can freely penetrate into the smooth muscle and endothelial cell layers. As DAB is poorly able to cross the blood-brain barrier and arterial wall, it is likely that this lack of detection may be related to limited distribution of Mn²⁺/DAB perfusate when applied to the subarachnoid space. Additionally, as the life of -O₂⁻ is considerably short, it remains possible that this species had already decayed in these regions before detection. Therefore, we cannot assuredly exclude the possibility that -O₂⁻ is produced in the media and endothelial cells of the vascular wall after SAH.

Regarding the cellular source(s) of -O₂⁻, they were particularly abundant around degenerated erythrocytes and, less so, near infiltrating macrophages and neutrophils. The aforementioned result indicates that -O₂⁻ is derived not only from autoxidation of oxyHb but also from infiltrating activated macrophages and neutrophils. In this regard, it is interesting to note that intrathecal injection of talc (crystallized hydrous magnesium silicate) in dogs induces severe foreign body (nonspecific inflammatory) reaction in the subarachnoid space accompanied by significant arterial narrowing and structural damage, thereby mimicking in many ways cerebral vasospasm after SAH, but without evidence of extravasated erythrocytes. These arterial changes were significantly attenuated by intrathecal administration of SOD, suggesting that foreign body reaction mediated via infiltrating macrophages and neutrophils may contribute to cerebral vasospasm.

Nonetheless, data presented herein support the hypothesis that -O₂⁻ as a result of autoxidation of extravasated erythrocyte-derived Hb is the primary contributor to the pathogenesis of cerebral vasospasm after SAH.

Finally, it seems likely that the main source of Fe²⁺ or Fe³⁺ iron products in this study is lysate from subarachnoid blood clots, although an alternate possibility is that some Fe²⁺ or Fe³⁺ iron ions were released from degenerating infiltrating macrophages during erythrophagocytosis, which is known to occur in SAH.

**Possible Pathogenetic Role of Intracisternally Generated -O₂⁻ and Fe²⁺/Fe³⁺ Iron Ions**

Mounting evidence suggests that oxyHb has a wide spectrum of biological actions that include the following: (1) generation of ROS, (2) a direct vasoconstrictive effect ex vivo, and (3) scavenging of NO. The chemical pathway whereby oxyHb leads to the ROS cascade together with Fe²⁺ or Fe³⁺ iron ions is based on the Haber-Weiss and Fenton reactions. The importance of the present study lies in the fact that it demonstrated the coexistence of -O₂⁻ with Fe²⁺ or Fe³⁺ iron ions. Such an environment certainly favors the occurrence of Haber-Weiss or Fenton reactions, leading to generation of the most harmful species of ROS, i.e., -OH. Furthermore, peroxynitrite generation as a consequence of the direct interaction between NO and -O₂⁻ may also be an important ROS contributor to the pathogenesis of SAH, as its formation is approximately 3.5 times faster than its dismutation by SOD. Collectively, ROS initiate oxidative damage of DNA, mitochondria, and, most importantly, membrane phospholipids. Lipid peroxidation, in turn, brings about the activation of membrane phospholipase A₂, release of arachidonic acid, intercellular accumulation of diacylglycerol, and activation of protein kinase C. Although the generation of -O₂⁻ in the present study was detected only in the adventitia of the vascular wall and subarachnoid space due to technical limitations, markedly increased levels of -O₂⁻ may cause an imbalance of endothelial vasoactivity (induction of vasoconstriction when the ratio of -O₂⁻ to NO is increased) as well as upregulation of adhesion molecules (such as intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]), leading to microcirculatory derangement. To conclude that ROS are primary in the pathogenesis of cerebral vasospasm after SAH, however, further experiments are warranted to detect ROS, including -O₂⁻, in the arterial wall. Nonetheless, the present study is the first step toward direct evidence of ROS in the pathogenesis of cerebral vasospasm after SAH.

Thus, on the basis of the aforementioned ROS cascade-promoting reactions, a variety of antioxidants such as SOD, the OH scavenger AVS [(+−−−−−−−−−−−N−N-propylenedinitramide), ebselen (an agent exhibiting glutathione peroxidase–like activity), the antioxidant aminoester, the singlet oxygen scavenger histidine, and iron chelators have been shown to be prophylactic against cerebral vasospasm in animal models as well as in SAH patients. Of particular interest is the Fe²⁺ chelator 2,2'-dipyridyl, which has been shown to completely prevent delayed vasospasm in a primate model of SAH, pointing to an important
role of the Fe$^{2+}$ ion in the pathogenesis of cerebral vasospasm, as ROS formation requires the ferrous moiety and Fe$^{2+}$ is a potent trapping agent for NO. In this regard, the histochemical evidence presented in this report may provide one mechanism to explain why the Fe$^{2+}$ chelator 2,2'-dipyridyl appears to prevent delayed vasospasm after SAH.

In conclusion, the present study provides histochemical evidence for enhanced production of O$_2^-$ and Fe$^{2+}$/Fe$^{3+}$ iron ions in the subarachnoid clot as well as in the adventitia of the arterial wall on days 2 and 7 after SAH. Thus, O$_2^-$ itself and subsequently generated ROS, particularly OH-, are considered to play a primary role in the pathogenesis of cerebral vasospasm after SAH.

Acknowledgment

This work was supported in part by grants from the Ministry of Education, Science, and Culture, Japan.

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Stroke. 2001;32:636-642
doi: 10.1161/01.STR.32.3.636
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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