Chemiluminescence in Hypoxic Brain — The Second Report: Cerebral Protective Effect of Mannitol, Vitamin E and Glucocorticoid

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SUMMARY The effect of vitamin E, betamethasone and mannitol upon a series of pathological free radical reactions within hypoxic brain tissue was evaluated by the chemiluminescence method. Hypoxia was induced by arterial hypoxemia (PaO₂ 17–22 mmHg) with normocapnia (PaCO₂ 28–38 mmHg) and normotension (MABP 100–140 mmHg). 4%O₂-96%N₂ mixed gas was used to obtain the lowered PaO₂.

In the untreated group, increased chemiluminescence was measured in the hypoxic state and the early stage of the initial post-hypoxic state. In the groups administered vitamin E, betamethasone, mannitol and a combination of them reduced chemiluminescence was detected.

To explore the reaction stage at which the drugs act in lipid peroxidation, chemiluminescence spectra was analyzed using the brain homogenate with the drugs added. Intensity peaks of the spectra were around 480, 520–530, 570, 620–640, 680–700 nm before addition of the drugs.

All the intensity peaks diminished after addition of vitamin E and betamethasone, but very little decrease occurred after mannitol.

The lowered chemiluminescence value may indicate the free radical scavenging action of vitamin E, betamethasone and mannitol in vivo. Chemiluminescence spectroanalysis shows that vitamin E and betamethasone act on the late chain reaction following hydroperoxide and mannitol acts on the early reaction — generation of active oxygen.

FIFTEEN YEARS AGO we performed a direct operation on an aneurysm of the right middle cerebral artery of a 50 year-old female, during which 50 minute temporary vascular occlusion at normothermia was unavoidable. Despite the occlusion, the postoperative recovery was uneventful and she was discharged without neurological symptoms. These events led us to suspect that the mannitol which had been administered to decrease intracranial pressure during the operation and prior to vascular occlusion had worked to prevent the development of cerebral infarction, which had been expected due to the lengthy vascular occlusion. Since then, we have been investigating the effects of mannitol on cerebral function using various animal models1, 2 for producing cerebral infarction in the dogs. Using one variation of the canine infarction model3 has been demonstrated the cerebral protective effects of mannitol using brain electrical activity as an index of the brain's functional state.4–6 Moreover, the protective effects of related substances have also been demonstrated, including those of vitamin E,7 glucocorticoid and perflurochemicals administered with the mannitol.8

Recently, we have used a chemiluminescence method9–12 together with a hypoxic rat brain preparation, and found a significant increase in photon emission, which is known to be a demonstration of a propagation in free radical reaction. In the present study, we have investigated the effects on the hypoxic brain of various cerebral protective drugs, including vitamin E,13 betamethasone14–20 and 20% mannitol1, 5, 6, 8 alone or in combination. Moreover, in order to determine the stage of the chemical reactions at which these chemicals have their scavenging effects during lipid peroxidation,21–25 we have performed chemiluminescence spectral analysis before and after drug administration using the brain homogenate at post-hypoxia-5 minutes.26–28

Study was also made of the capacity of the above drugs, administered prior to the production of the hypoxia, to maintain normal electrical activity of the rat brain even in a hypoxic state by means of comparison with a control group on EEG.

Materials and Methods

Administered drugs and doses included vitamin E, 30 mg/kg (α-tocopherol, 20 mg/ml solution from Eisai Co., Tokyo, Japan, diluted with 0.9% saline); betamethasone, 1 mg/kg (2 mg/ml solution from Shionogi Pharmaceutical Co., Osaka, Japan, diluted with 0.9% saline); and 20% mannitol 10 ml/kg.

One hundred and forty-five male Wistar rats each weighing 250–280 grams were used. In the chemiluminescence value study, the control group (0.5 ml of 0.9% saline was injected intravenously) were sacrificed as follows: 9 at pre-hypoxia, 5 at hypoxia — 3 min, 19 at hypoxia — 5 min, 9 at post-hypoxia — 5 min and 5 at post-hypoxia — 30 min. Fifteen animals received 30 mg/kg of vitamin E intravenously, 3 at pre-hypoxia, 6 at hypoxia — 5 min and 6 at post-hypoxia — 5 min. Twenty-three animals received 1 mg/kg of betamethasone intravenously — 3 at pre-hypoxia, 10 at hypoxia — 5 min and 10 at post-hypoxia — 5 min. Fourteen animals received 10 ml/kg of 20% mannitol intravenously — 3 at pre-hypoxia, 5 at hypoxia — 5 min and 6 at post-hypoxia — 5 min were
sacrificed. Twenty-one animals received combined administration of vitamin E (30 mg/kg), betamethasone (1 mg/kg) and 20% mannitol (10 ml/kg), namely, 3 at pre-hypoxia, 9 at hypoxia — 5 min and 9 at post-hypoxia — 5 min were sacrificed at each time interval (table 1).

In the study of chemiluminescence spectral analysis, ten animals at post-hypoxia — 5 min were used, namely, 3 served as the control and 2 for vitamin E, 2 for betamethasone and 3 for 20% mannitol. In the EEG study, ten animals served as the control and 5 received combined administration of vitamin E, betamethasone and 20% mannitol.

The rats were anesthetized by placing them in a container filled with 20%O₂ and 80%N₂. Under halothane anesthesia, they were given intravenous injections of 0.8 mg/kg pancuronium bromide. After tracheotomy and intubation, they were artificially respirated using a Harvard respirator. The femoral artery was cannulated for arterial blood gas analysis and measurement of blood pressure, and the femoral vein was cannulated for administration of the drugs.

A suitable mixture of O₂ and N₂ was made to flow to the respirator while arterial blood gases were analyzed in both the pre-hypoxic and post-hypoxic state. PaO₂ was kept at 110–140 mmHg, PaCO₂ at 35–45 mmHg and MABP at 100–140 mmHg. When blood pressure and arterial blood gases had become stabilized in a pre-hypoxic state, the drugs were administered intravenously (over 15 minutes in the case of 20% mannitol). Thirty minutes later, the hypoxic state was produced for 5 minutes.

This was done by instantaneous introduction of a 4%O₂ and 96% N₂ mixture into the respirator, while maintaining PaCO₂ within a normal range by decreasing the tidal volume — thereby preventing a fall in blood pressure. By this means, PaO₂ was reduced to 17–22 mmHg and PaCO₂ maintained at 28–38 mm Hg, MABP at 100–140 mmHg during the hypoxic state.

Following the above procedure, experiments were carried out only in those rats for which only PaO₂ was altered.

The skull bone was exposed through a skin incision over the parietal region to allow freezing of tissue in situ (Pon té et al, 1973) using a bottomless cup fas-

tened to the cranium. Liquid nitrogen was poured onto the brain at each time intervals. The brain tissue was kept at −80°C until analysis. The weight of tissue was constantly 500 mg in the chemiluminescence value study and diluted 10 fold in 0.9% saline using a teflon automatic homogenizer. The photons emitted from the homogenate were measured with a Chemiluminescence Analyzer (Model OX-7) from Tohoku Electronics Ltd. The chemiluminescence values were the means of 10 separate photon counts during 10 seconds in a period of 2.5–5 minutes following placement of the homogenate cell and heating to 35–36°C in a black box containing room air.

Chemiluminescence spectral analysis was made using 1.0 g of brain tissue at post-hypoxia — 5 min, which was diluted 10 fold in 0.9% saline and allowed to stand for 3–4 hours in room air at 35–36°C. When the chemiluminescence value had become "enhanced," i.e., had attained a level greater than 3000 counts/30 sec.g, it was possible to analyse using a filter spectrum analysis system (from the Inaba Laboratory of the Tohoku University Electronic Communication Research Institute).

This spectral analyzer has a set of colored glass filters which has the ability to cut off different sharp short-wavelengths. Vitamin E, betamethasone and 20% mannitol were individually dropped on the homogenate after it had been confirmed that there was no decrease in photon counts when the spectrum had been analyzed in the samples to which the drugs were not added. Spectral analysis was studied at 35–36°C in a black box containing room air. The amount of the drugs was 8 mg/g brain of vitamin E, 1.0 mg/g brain of betamethasone and 0.5 g/g brain of 20% mannitol.

For EEG monitoring, burr holes were opened bilaterally over the parietal lobe, and bipolar electrodes were placed epidurally. Comparison of the EEG records was made between the control group and the combined administration group.

The body temperature of the animals was monitored with a rectal thermometer and maintained at about 37°C.

### Experimental Results

**A. Chemiluminescence Value**

As we have previously reported,²⁹ the chemiluminescence values (mean ± SD) in the control animals were 231 ± 35 counts/10 sec.g (n = 5) after 3 minutes of hypoxia, 154 ± 62 counts/10 sec.g (n = 19) after 5 minutes of hypoxia and 217 ± 79 counts/10 sec.g (n = 9) at 5 minutes post-hypoxia. The pre-hypoxia (11 ± 15 counts/10 sec.g, n = 9) and the 30 minute post-hypoxia values (10 ± 13 counts/10 sec.g, n = 5) were close to zero. In contrast, in the rats administered vitamin E, virtually no photons were detected during pre-hypoxia, after 5 minutes of hypoxia, or at 5 minutes post-hypoxia (fig. 1-A) — pre-hypoxia 7 ± 6 counts/10 sec.g, (n = 3), hypoxia — 5 min 6 ± 13 counts/10 sec.g, (n = 6), post-hypoxia — 5 min 0 ± 0 counts/10 sec.g, (n = 6). Animals administered betamethasone had low photon counts at all periods, as follows: 17 ± 15 counts/10 sec.g (n = 3) in pre-

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**Table 1** Number of Sacrificed Animals with Administration of Various Drugs at Each Time Intervals in Chemiluminescence Value Study

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Vit. E</th>
<th>Betamethasone</th>
<th>Mannitol</th>
<th>Vit. E Betamethasone Mannitol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-hypoxia</td>
<td>9</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>3 min</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>19</td>
<td>6</td>
<td>10</td>
<td>5</td>
</tr>
<tr>
<td>Post-hypoxia</td>
<td>5 min</td>
<td>9</td>
<td>6</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>30 min</td>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>
CHEMILUMINESCENCE IN HYPOXIC BRAIN/Suzuki et al

hypoxia, 11 ± 16 counts/10 sec.g (n = 10) after 5 minutes of hypoxia and 32 ± 37 counts/10 sec.g (n = 10) after 5 minutes of post-hypoxia (fig. 1-B). Animals given 20% mannitol showed 0 ± 0 counts/10 sec.g (n = 3) in pre-hypoxia, 46 ± 37 counts/10 sec.g (n = 5) after 5 minutes of hypoxia and 4 ± 6 counts/10 sec.g (n = 6) after 5 minutes of post-hypoxia (fig. 1-C). When all three drugs were administered together, the following values were obtained: 3 ± 4 counts/10 sec.g (n = 3) in pre-hypoxia, 22 ± 40 counts/10 sec.g (n = 9) after 5 minutes of hypoxia and 4 ± 7 counts/10 sec.g (n = 9) after 5 minutes of post-hypoxia (fig. 1-D).

B. Chemiluminescence Spectral Analysis

Spectral peaks were found around at 480 nm, 520-530 nm, 570 nm, 620-640 nm and 680-700 nm in all of the untreated animals. The peaks which have greater intensity were around at 570 nm, 620-640 nm and 680-700 nm (figs. 2, 3, 4).

Administration of vitamin E or betamethasone resulted in a general inhibition of the intensity of the photon emission at all wavelengths (figs. 2, 3). In contrast, addition of mannitol resulted in the intensification of these peaks, without any of the inhibition produced by the other drugs (fig. 4).

C. EEG

Sequential study of the EEG was not able to be accomplished in all animals, because of sacrifice at different time intervals. Ten animals injected 0.5 ml of 0.9% saline served as controls and 5 received combined administration were used for sequential recording of the EEG. Typical pattern of the EEG results is depicted in fig. 5.

The EEG of the control showed the emergence of high voltage slow waves within 1 minute of producing the hypoxia and attenuation and nearly complete disappearance of the fast wave components after 4 minutes. After 5 minutes, the EEG record was predominantly delta waves. In the post-hypoxic state, continuous low
FIGURE 4. Effect of mannitol upon chemiluminescence spectra. Little decrease of the spectropeaks was shown after addition of mannitol.

voltage theta waves, which continued for 30 minutes, were seen.

In contrast, when vitamin E (30 mg/kg), betamethasone (1 mg/kg), and 20% mannitol (10 ml/kg) were administered together, there was the emergence of more high voltage slow waves at 2-3 minutes of hypoxic state than in the control group, and fast wave components were visible throughout the hypoxic state. In the post-hypoxic state, fast waves were seen after 30 seconds and after 10 minutes an EEG record similar to the pre-hypoxic state was found (fig. 5).

Discussion

In the first report of this research, we concluded that there is an increase in the chemiluminescence values in a rat brain homogenate sampled during a hypoxic state or sampled in an early post-hypoxic state. These increases in chemiluminescence levels are thought to be due to the generation and propagation of free radical reaction, which has progressed at least to the stage of production of alkyl radicals (R•). Since the measurement is made in air at 35-36°C, the air oxygen which comes into contact with the homogenate starts a chain reaction, as follows:

\[
R^* + O_2 \rightarrow ROO^*
\]

\[
RH + ROO^* \rightarrow ROOH + R^*
\]

which produces, in turn, a peroxy radical (ROO•), an alkyl radical (R•), and a hydroperoxide (ROOH). This is thought to result in a sequential increase in the volume of alkyl radicals (fig. 6).

In contrast, in the normal brain homogenate, there is little or no increase in chemiluminescence values over 30-40 minutes of contact with air oxygen — suggesting that there has not occurred pathological free radical reaction (fig. 7).

With regard to the mechanism of photon emission in chemiluminescence, it is known from the spectral peaks that singlet oxygen, particularly the Δg oxygen, is involved. Since the chemiluminescence measurements are made in the air, it is also known that the predominant singlet oxygen is produced by the breaking of the peroxy radical (ROO•) subsequent to the hydroperoxide and alkyl radical (ROOH and R•).

This experiment was attempted to make clear the effect of vitamin E, glucocorticoid and mannitol as free radical scavenger in lipid peroxidation. Vitamin E is known as an important free radical scavenger, due to its lipid solubility and occurrence in membranes. The protective effect of vitamin E is due to its ability to scavenge free radicals, such as superoxide anion or singlet oxygen, as well as its ability to scavenge lipid peroxy radicals, such as methyl linoleate radical.

Glucocorticoid is widely used for brain edema due to its stabilizing effect of cell membrane. Its pharmaceutical mechanism has been reported as suppressor of extrication of arachidonate acid by impeding phospholipase A2. The present data of chemiluminescence and its emission spectroanalysis are able to interpret the mechanism of glucocorticoid as radical scavenger in the breakdown of lipid hydroperoxide besides impeding phospholipase A2. So far some reports on glucocorticoid as free radical scavenger or inhibitor of autoxidation has published. Suzuki and Yagi (1973) reported that pretreated dexamethasone decreased the levels of edema and lipoperoxide products in cryogenic injury rat brain using the thiobarbituric acid assay. Seligman and Demopoulos (1979) have shown that glucocorticoid significantly decreased lipoperoxide products on UV irradiation-irritated liposome membrane in vitro.

Mannitol has been widely used to decrease cerebral volume and it has been reported to increase cerebral blood flow. On the other hand, Seki (1983) reported that mannitol caused an increase of rCBF...
A chemical compound with OH groups tends to combine with hydroxy radical and mannitol has a number of OH groups in its molecular structure.

In the present study on hypoxic brain significant decrease of chemiluminescence value at each time interval was found in the groups administered vitamin E, betamethasone, 20% mannitol and combination of three drugs. These findings are thought to indicate an in vivo free radical scavenging action of these drugs.

The spectral analysis of very weak light signals is accomplished with the successive insertion of colored glass filters arranged on a rotating disc into the optical path between the reflector and the photomultiplier. Twenty-seven colored filters are employed to cover the total wavelength region between 275 and about 680 nm with the transmission ranging from 45% to 60%.

It is well-known that chemiluminescence spectra which have been obtained from tissue homogenates placed in the air at 35–36°C have a greatly enhanced chain reaction from the hydroperoxide (ROOH). Consequently, the decreased spectral intensity found after application of vitamin E or betamethasone is thought to indicate that the peroxidative stage of the scavenging action by these drugs is subsequent to the hydroperoxide. In contrast, the fact that application of 20% mannitol did not result in significant decrease in the spectral intensity indicates that the reaction at which mannitol has its scavenging effect is in the early stage of lipid peroxidation. This finding is in agreement with previous reports that mannitol has a quenching effect on the hydroxy radical (OH•) only.

Electroencephalography is a useful means for evaluating the physiological function of the brain. In this experiment, the EEG of the control group was virtually flat during the hypoxic state, which has made it impossible to evaluate the effects of the drugs administered individually.

Nonetheless, a significant difference between the control group EEG record and that of the group administered all three drugs was found. In the control group, fast wave components disappeared during the hypoxic state, while in the post-hypoxic state theta waves continued to be seen. In contrast, in animals given all three drugs together, fast wave components were still seen
after 5 minutes of hypoxia and after 10 minutes in the post-hypoxic state, the EEG was similar to that of the prehypoxic state.

These findings on the electrical activity of the brain indicate that combined treatment with three drugs was effective in allowing for the recovery of cerebral function following hypoxia.

Conclusion

1. In experiments on the hypoxic brain using the rat, pre-treatment with vitamin E, betamethasone and 20% mannitol was found to decrease the chemiluminescence in the hypoxic brain tissue samples. These findings are thought to indicate free radical scavenging action of these drugs.

2. By means of chemiluminescence spectral analysis, it was found that vitamin E and betamethasone have their effects on the late stages of lipid peroxidation subsequent to the lipid hydroperoxide (ROOH). In contrast, mannitol has its quenching effect in the earlier stages of lipid peroxidation.

3. EEG recordings also indicated that combined pretreatment with vitamin E, betamethasone and 20% mannitol was effective in improving the recovery of brain electrical activity subsequent to hypoxia.

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22. By means of chemiluminescence spectral analysis, it was found that vitamin E and betamethasone have their effects on the late stages of lipid peroxidation subsequent to the lipid hydroperoxide (ROOH). In contrast, mannitol has its quenching effect in the earlier stages of lipid peroxidation.
23. EEG recordings also indicated that combined pretreatment with vitamin E, betamethasone and 20% mannitol was effective in improving the recovery of brain electrical activity subsequent to hypoxia.
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