EFFECT OF OXYGEN AT HIGH PRESSURE ON THE GROWTH OF EHRLICH ASCITES TUMOR; ESPECIALLY ON THE COMBINED THERAPY WITH METHYL-BIS-(2-CHLOROETHYL)AMINE N-OXIDE HYDROCHLORIDE

Tsuyoshi Kito
(1st Department of Surgery, Nagoya University School of Medicine*)

Synopsis

Effect of oxygen at high pressure (OHP) and methyl-bis(2-chloroethyl)amine N-oxide hydrochloride (HN₂-O) on the growth of hypotetraploid Ehrlich ascites tumor was investigated using 2-4 atm. abs. oxygen and 2 mg/kg of HN₂-O. The parameters for response were chiefly total tumor cell number in the abdominal cavity of mice, tumor weight inoculated into the back of mice, and survival time.

Four atm. abs. of oxygen alone decreased the total number of tumor cells and tumor weight, and prolonged survival time. A combination of OHP (3 and 4 atm. abs.) and HN₂-O decreased the total number of tumor cells and tumor weight. Antitumor effect was not observed in all groups treated with 2 atm. abs. oxygen. The mice treated with 3 and 4 atm. abs. oxygen exhibited a decrease in body weight. In histological observation, histological change in lung and heart was not noted in the mice treated with OHP.

The present experiments show that 4 atm. abs. OHP alone has antitumor effect and also that inhibitory effect of combination of HN₂-O and OHP (3 and 4 atm. abs.) on the tumor growth is more marked than that of treatment with HN₂-O alone.

INTRODUCTION

In treatment of malignant tumor, combined therapeutic measures have been receiving universal interest. The radiologists have long been aware that the effect of ionizing radiation can be enhanced by increasing the tissue oxygen tension. In 1936, the dependency of radiosensitivity on tissue oxygen tension was first demonstrated by Mottram.35) In 1953, Gray23) reinvestigated and confirmed the validity of this observation. Subsequent laboratory studies14, 18, 41, 42, 44) and clinical trials12, 13, 15, 38, 46, 47) have substantiated the evidence for potentiation of ionizing radiation by oxygen at high pressure (OHP).

Because the cytological actions of alkylating agents are similar to those of ionizing irradiation,3, 7, 8) several investigators have tested the effect of OHP and chemotherapy in the laboratory4-6, 9, 15, 17, 21, 28-32, 34) and in clinical cases.1, 2) However, the report in the literature on the effect of OHP and chemotherapy is inconclusive. The experiments reported here were designed to find the effect of OHP when used alone and in combination with nitrogen mustard N-oxide (HN₂-O) and to decide the optimum pressure of OHP.

* Tsurumai-cho, Showa-ku, Nagoya (紀藤 郎).
MATERIALS AND METHODS

Animals used throughout these experiments were female SMA mice about 70 days old, obtained from the Supplying Center of Laboratory Animals in this medical school. They were fed with a standard pellet diet and given drinking water ad libitum.

Tumor cells used in this study were Ehrlich hypotetraploid stock (Kaziwara 4n) maintained in adult female SMA mice through serial intraperitoneal transplantation at 7-day intervals in this laboratory.

In the 1st series of experiments, the mice were inoculated intraperitoneally with 0.2 ml of the cell suspension containing $5 \times 10^6$ viable cells as determined by the Schreck test and were sacrificed on the 7th day after inoculation. Then the total number of tumor cells was calculated for each mouse by the rinse technique and the mitotic index of the tumor cells in the smears of ascites fluid, stained with Giemsa, was obtained. In another experiment, survival time of the mice was observed.

The 2nd series of experiments involved the subcutaneous injection of ascites tumor, in which 0.1 ml of tumor ascites fluid ($5 \times 10^6$ cells) was inoculated subcutaneously into the back of mice. On the 11th day, these mice were sacrificed, and any solid tumor which developed subcutaneously was excised and weighed.

After tumor inoculation, all experimental mice were divided randomly into 4 groups. One group served as a control at atmospheric pressure in each experiment. The 2nd group received only high pressure oxygen, as follows: On the day of tumor inoculation, animals were placed in a pressure chamber. The pressure was maintained for 1 hr. each day for the subsequent 6 days in the case of intraperitoneally inoculated mice and for 10 days in the case of subcutaneously inoculated mice. High pressure oxygen was given at 2, 3, or 4 atm. abs.

Fig. 1. Pressure chamber
EFFECT OF OHP ON THE GROWTH OF EHRlich ASCITES TUMOR

The pressure chamber was developed in this department by Sakakibara et al., and measures 650 × 1115 mm, as shown in Fig. 1. The chamber can be ventilated by means of four valves for removal of accumulated carbon dioxide. This chamber was used for the hyperbaric experiments with pure oxygen. Fifteen minutes were necessary before the internal pressure reached 4 atm. abs., 10 mins. for 3 atm. abs., and 7 mins. for 2 atm. abs. Before 2 atm. abs. was attained, oxygen was insufflated with one valve half open, in order to replace the air fully in the chamber. After the desired pressure of the chamber was reached, it was maintained for 1 hr. and then decompressed to the atmospheric pressure taking 30 mins. from 4 atm. abs., 20 mins. from 3 atm. abs., and 15 mins. from 2 atm. abs., to prevent bubble formation of oxygen in the blood.

The 3rd group received only cancer chemotherapy. Intraperitoneal injection of HN₂-O (2 mg/day/kg of body weight of the mice) was started on the day following tumor inoculation and terminated on the 6th day in the case of the intraperitoneally inoculated mice, and 10th day in the case of subcutaneously inoculated mice.

The 4th group received both OHP and chemotherapy. OHP was treated at the same pressure level and for the same period as that of the group treated with OHP alone. HN₂-O was injected with the same procedure as that of the group treated with HN₂-O alone.

In the control and OHP groups, 0.1 ml of isotonic saline was injected intraperitoneally. After sacrifice of mice, the heart and lung were fixed in formol, embedded in paraffin, and were stained with Hematoxylin-Eosin.

RESULTS

Effect of OHP and HN₂-O on the Growth of Ehrlich Ascites Tumor in the Abdominal Cavity of Mice The total number of tumor cells per mouse was calculated on the 7th day after intraperitoneal inoculation.

In the series of 4 atm. abs. OHP, the growth of tumor in the abdominal cavity of mice was inhibited by treatment with OHP. Table I shows that the difference of the total tumor cell number between the control mice and those treated with OHP alone and also the difference between the mice treated with HN₂-O alone and those treated with HN₂-O and OHP are statistically significant (P<0.001).

Table II shows the result in the series of 3 atm. abs. OHP treatment. The difference between the control mice and those treated with OHP alone was not significant while

Table I. Effect of 4 Atm. Abs. OHP and HN₂-O on the Growth of Ehrlich Ascites Tumor in the Abdominal Cavity of Mice

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Tumor cell No./mouse (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>6.50 × 10⁴ ± 0.39</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OHP</td>
<td>8</td>
<td>3.98 × 10⁴ ± 0.25</td>
<td></td>
</tr>
<tr>
<td>HN₂-O</td>
<td>8</td>
<td>3.00 × 10⁴ ± 0.03</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OHP + HN₂-O</td>
<td>8</td>
<td>2.05 × 10⁴ ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>

Evaluation of statistical difference was based on the Student's t-test.
the difference between the mice treated with HN$_2$-O alone and those treated with HN$_2$-O and OHP was statistically significant (P<0.001). In the series of 2 atm. abs. OHP treatment, as shown in Table III, the difference between the control mice and those treated with OHP alone, and also the difference between the mice treated with HN$_2$-O alone and those treated with HN$_2$-O and OHP were not significant.

**Table II. Effect of 3 Atm. Abs. OHP and HN$_2$-O on the Growth of Ehrlich Ascites Tumor in the Abdominal Cavity of Mice**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Tumor cell No./mouse (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15</td>
<td>6.68×10^4±0.21</td>
<td></td>
</tr>
<tr>
<td>OHP</td>
<td>15</td>
<td>6.33×10^4±0.15</td>
<td></td>
</tr>
<tr>
<td>HN$_2$-O</td>
<td>15</td>
<td>3.20×10^4±0.20</td>
<td></td>
</tr>
<tr>
<td>OHP+HN$_2$-O</td>
<td>15</td>
<td>1.74×10^4±0.15</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Effect of OHP and HN$_2$-O on the Growth of Solid Ehrlich Ascites Tumor in the Subcutaneous Space of Mice The solid tumor that developed subcutaneously was excised and weighed on the 11th day.

In the series of 4 atm. abs. OHP treatment, a statistical analysis showed a significant difference (P<0.001) in the growth of solid tumor between the control mice and those treated with OHP alone. The difference of tumor weight between the mice treated with HN$_2$-O alone and those treated with HN$_2$-O and OHP was also statistically significant (P<0.001) (Table IV).

**Table IV. Effect of 4 Atm. Abs. OHP and HN$_2$-O on the Growth of Solid Ehrlich Ascites Tumor**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Tumor wt. (mg) (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>232.7±10.8</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OHP</td>
<td>7</td>
<td>173.4±36.0</td>
<td></td>
</tr>
<tr>
<td>HN$_2$-O</td>
<td>7</td>
<td>158.7±14.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OHP+HN$_2$-O</td>
<td>7</td>
<td>129.7±21.0</td>
<td></td>
</tr>
</tbody>
</table>
EFFECT OF OHP ON THE GROWTH OF EHRLICH ASCITES TUMOR

In the series of 3 atm. abs. OHP treatment, the average tumor weight in the group treated with HN$_2$–O and OHP was less than that in the group treated with HN$_2$–O alone, its difference between them being statistically significant (0.02<P<0.05) (Table V).

In the series of 2 atm. abs. OHP treatment, no difference in the tumor weight was observed (Table VI).

### Table V. Effect of 3 Atm. Abs. OHP and HN$_2$–O on the Growth of Solid Ehrlich Ascites Tumor

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Tumor wt. (mg) (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>134.0±17.5</td>
<td></td>
</tr>
<tr>
<td>OHP</td>
<td>7</td>
<td>118.7±18.7</td>
<td></td>
</tr>
<tr>
<td>HN$_2$–O</td>
<td>7</td>
<td>118.6±25.5</td>
<td>0.02&lt;P&lt;0.05</td>
</tr>
<tr>
<td>OHP+HN$_2$–O</td>
<td>7</td>
<td>56.6±6.2</td>
<td></td>
</tr>
</tbody>
</table>

### Table VI. Effect of 2 Atm. Abs. OHP and HN$_2$–O on the Growth of Solid Ehrlich Ascites Tumor

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Tumor wt. (mg) (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>369.3±76.5</td>
<td>0.4&lt;P&lt;0.5</td>
</tr>
<tr>
<td>OHP</td>
<td>7</td>
<td>287.9±55.4</td>
<td></td>
</tr>
<tr>
<td>HN$_2$–O</td>
<td>7</td>
<td>176.4±24.3</td>
<td>0.05&lt;P&lt;0.1</td>
</tr>
<tr>
<td>OHP+HN$_2$–O</td>
<td>7</td>
<td>156.4±1.7</td>
<td></td>
</tr>
</tbody>
</table>

Effect of OHP and HN$_2$–O on the Survival Time of Intraperitoneally Tumor-inoculated Mice  Table VII shows the mean survival time of mice in each treated group and Fig. 2 shows the mean survival rate in the series of 4 atm. abs. OHP treatment. A statistically significant increase (0.02<P<0.05) in the survival time was observed in the group treated with OHP alone as compared with the control group.

### Table VII. Effect of 4 Atm. Abs. OHP and HN$_2$–O on the Survival Time of Tumor-inoculated Mice in the Ascites Form

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Survival time (days) (Mean±S.E.)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>19.6±0.6</td>
<td>0.02&lt;P&lt;0.05</td>
</tr>
<tr>
<td>OHP</td>
<td>8</td>
<td>21.1±0.4</td>
<td></td>
</tr>
<tr>
<td>HN$_2$–O</td>
<td>8</td>
<td>23.6±1.0</td>
<td></td>
</tr>
<tr>
<td>OHP+HN$_2$–O</td>
<td>8</td>
<td>23.9±1.3</td>
<td></td>
</tr>
</tbody>
</table>
Table VIII and Fig. 3 show the survival time of mice in the series of 3 atm. abs. OHP treatment. A slight increase in the survival time was observed between the mice treated with \( \text{HN}_2\text{O} \) alone (4 mg/kg of body weight) and those treated with \( \text{HN}_2\text{O} \) and OHP, though the difference was not significant.
Influence of OHP on the Body Weight of Tumor-bearing Mice  During oxygen therapy in the series of 3 and 4 atm. abs. OHP treatment, loss of body weight in mice was observed in the two groups treated with OHP, but the mice regained their body weight within a week after the cessation of pressurization (Fig. 4).

Effect of OHP and HN₂-O on the Mitosis of the Tumor Cells in the Abdominal Cavity of Mice Smears of ascites fluid, stained with Giemsa solution, were prepared from the mice in each treated group. A slight decrease in the mean mitotic index was observed in the three groups treated with OHP alone, HN₂-O alone, and OHP combined with HN₂-O as compared with the control group (Table IX).
Influence of Oxygen Toxicity on the Lung and Heart of Mice

Microscopic examination revealed that there was no harmful change due to the oxygen toxicity in the lung and heart of mice treated with OHP.

**DISCUSSION**

The clinical utilization of air at high pressure can be traced back to the 19th Century when it was used for the treatment of caisson disease. This attracted the interest of some investigators and Boerema brought this old technique to a new clinical application when he used oxygen at high pressure (OHP) in cardiac surgery in 1956. The hyperbaric chamber was developed in this department by Sakakibara et al.

The early reports of Warburg that suggested the possible importance of tissue anoxia and anaerobic metabolism in the development of cancer cells stimulated further investigation. The first attempt used high pressure oxygen on cancerous and normal tissues was that of Fischer in 1926. De Almeida found in 1934 that OHP had a very definite cancerocidal action on fuso-cellular sarcoma of rats.

Gray refocussed attention on the earlier finding of Mottram and showed that the sensitivity of tumor cells to X-rays was about three times greater when they were irradiated in a well-oxygenated medium as under anoxic conditions. It has been reported that the effect of ionizing irradiation could be enhanced by increasing the tissue oxygen tension in animals and in humans. Churchill-Davidson treated a number of patients with irradiation and OHP. The importance of oxygen tension to radiosensitivity is now well recognized. Because ionizing irradiation and alkylating agents have similarities in cytological response, the present study was undertaken to investigate the effect of OHP and HN₂-O on Ehrlich ascites tumor.

Polarographic technique for the measurement of tissue oxygen tension has shown a markedly reduced pO₂. Cater et al. found by exposure of mice and rats to OHP that optimum elevation in tissue oxygen tension for maximum radiosensitization was achieved.

It was demonstrated by many workers that antitumor effect of chemotherapy was enhanced with OHP, whereas some investigators observed no stimulatory effect of OHP on chemotherapy. Marshall et al. found that there was a decrease in LD₅₀ with a corresponding increase in toxicity of nitrogen mustard, under conditions of both hypoxia and hyperoxia. It was found that OHP alone had antitumor effect, though Campbell and McCredie et al. indicated no effect of OHP alone.

---

**Table IX. Effect of 3 Atm. Abs. OHP and HN₂-O on the Mitosis of Tumor Cells in the Abdominal Cavity of Mice**

<table>
<thead>
<tr>
<th>Study groups</th>
<th>No. of mice</th>
<th>Mitotic index (%) (Mean±S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>11.5±0.8</td>
</tr>
<tr>
<td>OHP</td>
<td>8</td>
<td>9.5±0.7</td>
</tr>
<tr>
<td>HN₂-O</td>
<td>8</td>
<td>8.5±0.7</td>
</tr>
<tr>
<td>OHP+HN₂-O</td>
<td>8</td>
<td>9.0±0.8</td>
</tr>
</tbody>
</table>
Present data indicate that treatment with 4 atm. abs. OHP inhibits the growth of tumor to some extent, and that the combination of OHP (3 and 4 atm. abs.) and HN₂-O inhibits more markedly the tumor growth than does HN₂-O alone.

In view of high anaerobic glycolysis in tumor cells, these results can be explained in part by postulating that tumor cells have a higher sensitivity to OHP than normal cells. Gerschman pointed out a partial common mechanism in OHP and ionizing irradiation. On the other hand, Okada reported that OHP elevated host resistance in experiments with rats. For these reasons, it is considered that OHP has an antitumor effect and also a beneficial influence on the tumor-bearing host.

Alkylating agents have an effect on the tumor which is similar to that of ionizing irradiation. From this, it is inferred that the tumoricidal effect of such drugs will be enhanced when the tumor is hyperoxygenated. HN₂-O was selected because of its short biological half-life.

The animals treated with 3 and 4 atm. abs. OHP exhibited a decrease in body weight during OHP treatment. This weight loss may be due to an oxygen toxicity. However, the animals regained weight within a week after cessation of pressurization. In the histological findings no evidence of the lung and heart damage was observed in the mice treated with OHP.

The present experiments show that 4 atm. abs. OHP alone has an antitumor effect and also that inhibitory effect of combination of OHP (3 and 4 atm. abs.) and HN₂-O on the tumor growth is more marked than that of treatment with HN₂-O alone. Mori observed that the rats treated with 4 atm. abs. OHP had some evidences of the lung and heart damage, though no harmful change was seen in the present study. Because the exposure to higher pressure causes greater oxygen toxicity, it is considered that 3 atm. abs. OHP is more applicable when OHP is used with HN₂-O.

The exact mechanism of the action of OHP on the growth of tumor and on the HN₂-O treatment cannot be deduced from the data obtained in the present experiments. However, the present study indicates that OHP treatment has an antitumor effect on the tumor growth to some extent and that a more adequate treatment with OHP, combined with other antitumor agent, might bring about much more inhibitory effect on the tumor growth.

The author wishes to express his thanks to Prof. Yoshio Hashimoto, Dr. Kinsaku Sakakibara, and Dr. Tatsuo Hattori, all of this Department, for their helpful advice and encouragement throughout the present study, and also to Prof. Jun Takeuchi, Department of Pathology, School of Dentistry, Aichi-Gakuin University, for fruitful discussion of the problems.

References

T. KITO