Examination of the influence to give a hypoxic ischemic encephalopathy by atmospheric pressure plasma and umbilical cord blood

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In recent years, atmospheric-pressure plasmas are applied in the medical applications. Although the mechanism of action remains unclear, new biomedical applications of plasma have been found. Experiments using atmospheric pressure plasmas confirmed several effects such as burn healing with angiogenesis. In addition, umbilical cord blood is expected to be applied to the field of regenerative medicine due to the presence of mesenchymal stem cells. We have been focusing treatment of Hypoxic Ischemic Encephalopathy (HIE). HIE caused by the discontinuation of blood supplied means that a part of the brain is necrotized, and a brain function is impaired. There is no fundamental therapy for HIE except for symptomatic therapy as of now. Because of preservation and recovery of brain functions can be expected, as a study on HIE treatment, we consider and report on a method of administrating atmospheric-pressure plasma and umbilical cord blood to HIE model rat.

Keywords: Hypoxic Ischemic Encephalopathy, atmospheric-pressure plasmas, umbilical cord blood, CT.

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1. Background

In recent years, we have applied atmospheric pressure low temperature plasma has to medical applications. Although the mechanism of action is still unknown, we have found a new biomedical application of plasma. In experiments using atmospheric pressure low temperature plasma, we confirmed several effects such as burn wound healing by angiogenesis [1,2]. In addition, we expect that umbilical cord blood containing mesenchymal stem cells will be applied to the field of regenerative medicine [3]. We have focused on the treatment of hypoxic ischemic encephalopathy (HIE). HIE caused by discontinuation of supplied blood means that part of the brain is necrosed and brain function is impaired. There is no fundamental cure for HIE other than current symptomatic treatment. Regarding the treatment of HIE, we predict conservation and recovery of brain function. Therefore, we administer the atmospheric pressure low temperature plasma and umbilical cord blood in combination with HIE model rats, and report the results obtained.

2. What is hypoxic encephalopathy

We refer to the state in which consciousness disorder and respiratory disorder are observed in neonatal period in general as neonatal encephalopathy. Neonatal encephalopathy occurs in about 1 to 6 people for 1000 births, 50 to 80% of which are HIE [4]. HIE is a brain disorder caused by falling into a hypoxic state due to oxygen deficiency in the blood and an ischemic state caused by a decrease in cerebral blood flow. Brain oxygen consumption corresponds to about 20% of total body oxygen consumption, the brain is an organ requiring oxygen in particular. Therefore, the brain is vulnerable to hypoxia. The cerebral cortex, hippocampus, cerebellum are particularly vulnerable to hypoxic conditions and may be damaged or obstructed. When the brain tissue is damaged, obstacles remain in motor function and memory function, which is the function of the site, depending on the damaged or injured part of the brain. As symptomatic treatment, brain cryotherapy that keeps body temperature low to prevent progress of the disorder, artificial respiration management to administer oxygen for improving hypoxic conditions, etc. exist. But there is no effective treatment method.

3. What is atmospheric pressure low temperature plasma

Matter usually exists in what is called three states of solid, liquid, and gas. When the energy is low and the most stable state is solid, when the energy becomes high, it becomes liquid, gas and plasma. Plasma is said to be the "fourth state" following the three states of matter. Plasma is a state in which atoms or molecules are ionized. In the plasma, cations with positive charges and electrons with negative charges are present in almost equal amounts. Ionization means that atoms and molecules are divided into cations and electrons. Plasma is defined as a medium that maintains an average quasi-neutral state. Quasi-neutral means that cations and electrons are nearly equal, so they are electrically neutral. When plasma is generated near the atmospheric pressure, a violent collision between electrons, cations, and neutrons occurs, exchange of energy is performed at high speed and shifts to thermal equilibrium. Thermal equilibrium means that the energy is given by collision between particles, so that the
temperature of cations and neutrons rises and the temperatures of electrons, cations, and neutrons become equal. The thermal equilibrium plasma has high temperature of about 10000 K at atmospheric pressure and it is called high temperature plasma. In the case of generating in the vicinity of low atmospheric pressure, non-thermal equilibrium is obtained because collisions between particles are small. The non-thermal equilibrium plasma is called low-temperature plasma because the temperature of cations and neutrons is kept at about room temperature. However, even under atmospheric pressure, it becomes non-thermal equilibrium by controlling so as not to be thermally activated under special conditions, resulting in low temperature. The generation method that dielectric barrier discharge or plasma jet is given as an example.

In this research, we use the plasma generator shown in Chapter 6 to generate atmospheric pressure low temperature plasma. In the plasma generation part, a cylinder of a dielectric (quartz tube) is used, electrodes are coaxially installed inside and outside, and a high voltage is applied to generate plasma. It has been confirmed that the atmospheric pressure low temperature plasma that can be generated in this apparatus keeps the body surface temperature of the rat at about 40 degrees during irradiation. Since the average body temperature of rats is about 38 degrees, the atmospheric pressure low temperature plasma used in this study can be said to be about 40 degrees [5].

4. Method for preparing HIE model rat

In this study, it is necessary to prepare model rats that developed HIE, so we made HIE model rats. We used 1 week old newborn rats (wistar, SPF) for this treatment. Since rodents such as rats are resistant to hypoxic environments, we performed a hypoxic load on rats with an ischemic load [6]. The details of the procedure are shown below.

In the experiment, we anesthetized to stop the rat’s body movement. We performed general anesthesia using sevoflurane against rats.

After induction of anesthesia, we performed left common carotid artery ligation to apply ischemic load. In order to ligate the left common carotid artery, the neck was incised first. Next, the left common carotid artery existing around the respiratory tract was confirmed and lifted using the first bending forceps. The suture was passed under the lifted left common carotid artery and the left common carotid artery was ligated twice. Thereafter, the incised neck portion was sutured.

We performed low oxygen loading on rats by creating a low oxygen environment and exposing rats. We used an incubator to reduce hypoxia. We housed rats in an incubator and exposed for 2 hours in a low oxygen environment with an air temperature of 37 °C, oxygen concentration of 8% and nitrogen concentration of 92%. Fig. 1 shows schematic diagram of low oxygen load environment manufacturing apparatus.

Fig. 1 Schematic diagram of low oxygen load environment manufacturing apparatus.

5. Review of administration site

We examined the site to administer the compound of atmospheric pressure low temperature plasma and umbilical cord blood to the prepared HIE model rats. In order to investigate the effect of treating HIE of composite of atmospheric pressure low temperature plasma and umbilical cord blood, examination of the administration site was necessary. We considered intra-ventricular administration that is expected to be absorbed into the vascular system close to the target organ, the brain. The ventricles refer to the lateral ventricle, the third ventricle, and the fourth ventricle. It is said that the cerebral ventricle fluid circulates in the ventricle and there is no difference in administration to each ventricle. Generally, ventricular fluid circulates in the order shown below. Lateral ventricle, Monroe hole, third ventricle, midbrain waterway, fourth ventricle, Ruska pore, mandibaldi hole, subarachnoid space. In addition, ventricular fluids are exchanged with blood by arachnoid granules and capillaries on the brain parenchyma. In general, intracerebroventricular administration is performed in the third ventricle or the lateral ventricle other than the fourth ventricle, which is in the deep region and highly dangerous. Therefore, intracerebroventricular cord blood administration experiments to the third / lateral ventricle were per-formed in HIE model rats.

Immediately after production of model rats, a mixture of 0.05 mL of umbilical cord blood and heparin, physiological saline was administered to the third / lateral ventricle. The administration was carried out from above the scalp using a 27 G injection needle. As a result, the survival rate in the third intraventricular administration was 56%, and the survival rate in the lateral ventricle administration was 63%. Considering the size of the individual, 27G was used for the injection needle used for administration. If a larger needle (one with a smaller gauge number) is used for administration, the following influences are conceivable. For example, it is considered that the mortality rate will rise because the possibility that hurting the brain and the possibility of damaging the blood vessels in the brain increases. Therefore, umbilical
cord blood administration to the third / lateral ventricle was considered to be possible to survive the subject sufficiently for examining the therapeutic effect of HIE. However, the survival rate in the third intracerebroventricular administration was lower than that in the lateral ventricle. The reason for this is considered to be that there was an individual who died of bleeding that occurs when the needle is pulled because the third intracerebroventricular administration is administered through the sagittal sinus. In view of the above, in this article we examined the lateral ventricular administration to HIE model rats. Fig. 2 shows schematic diagram of administration to the lateral ventricle.

6. Method for making plasma irradiation water

We generated a plasma by applying pulse voltage to helium gas. We used Voltage amplifier (HVPS: High Voltage Power Supply, HAVA 4321, manufactured by NF Circuit Design Block Co., Ltd.) and Gas Flow Controller (MFC: Mass Flow Controller, FCST1005FC -4F2-F1L-H, manufactured by Kofurok Co., Ltd.). When we generated plasma, helium gas was introduced into the flow path from the helium gas cylinder to the plasma generation section. We used a 8 mm plasma generator and a glass capillary with a tip inner diameter of 1 mm for the atmospheric pressure low temperature plasma generation. We introduced a 1 mm diameter tungsten electrode into a glass capillary. We introduced a 1 mm diameter tungsten ground electrode outside the glass capillary. Table 1 shows the generation conditions of atmospheric pressure low temperature plasma.

We injected 1.6 mL of physiological saline into a plastic container (15 W × 10 × D 20 H mm). We used a parafilm (plastic paraffin film) to cover the container to measure nitrogen oxides produced by the plasma in the gas phase inside the vessel. We opened a hole in the lid that was large enough to contain only the tip of the plasma irradiation device. We conducted plasma irradiation at 5 mm above the liquid level. Fig. 3 shows a conceptual diagram of experimental treatment with physiological saline and atmospheric pressure and low temperature plasma.

Using the experimental system described above, we have investigated the production of nitrogen oxides by plasma irradiation and the dissolution of nitrogen oxides produced by plasma irradiation in physiological saline. And using same system the plasma generation condition that the amount of product increased by plasma irradiation was obtained [7].

In addition, we confirmed that the content of nitrogen oxide, which is harmful to living organisms when incorporated in excess, is a value of 10 mg / L or less, which is the water quality standard value [8]. In addition, similarly to the umbilical cord blood administration experiment, we administered a dose of 0.05 mL of plasma-irradiated physiological saline.

We confirmed the survival of all the individuals administered in plasma irradiation physiological saline administration experiment. In addition, as individual symptoms of the administered individuals, individuals with smaller brain edema than the control group were confirmed, suggesting the effect of symptomatic reduction by plasma irradiation water.

7. Combined administration method of plasma irradiation saline and umbilical cord blood

We examined the effects of plasma irradiated water and umbilical cord blood on HIE model rats and survival rate by administration. We mixed 0.025 ml each of plasma irradiated saline and umbilical cord blood to create a composite. We administered 0.05 ml of a mixture of plasma-irradiated water and umbilical cord blood to the lateral ventricle from above the skin to the HIE model rat.
immediately after the preparation procedure. In addition, as a control group, 0.05 ml of a mixture of untreated saline and umbilical cord blood was similarly administered into the lateral ventricle and compared. As a result of our experiments, all individuals survived in both groups receiving the compound.

8. Examination of the effect of administration of plasma irradiated physiological saline and umbilical cord blood complex

We examined the effect of administration of the conjugate by CT assessment at 4 weeks of age, pathology dissection and brain appearance assessment.

8.1 Evaluation using conventional CT

We performed image evaluation of HIE model rats using X-ray CT apparatus for laboratory animals (Latheta LCT-200, Hitachi Aloka Medical Co., Ltd.). When HIE develops, it is evident that gray matter cerebral cortex, hippocampus, cerebellum are vulnerable to hypoxic conditions and it is easy for damage to remain. Therefore, we performed CT imaging with the CT imaging range as the vicinity around the cerebral cortex.

We evaluated the individuals who prepared the model rats at 1 week of age using CT after induction of anesthesia at 4 weeks of age. For anesthesia, 0.2 ml per 100 g body weight of rat was used as mixed anesthesia of three kinds mixed with dormitol (medetomidine hydrochloride), dolmicam (midazolam), bittlefare (butorphanol tartrate), and physiological saline. Shooting conditions for conventional CT are shown in the Table 2. Fig.4 shows the position and range of CT imaging. Using the these method, we evaluated the experimental group and control groups.

Table 2  CT imaging conditions.

<table>
<thead>
<tr>
<th>Voxel size (μm³)</th>
<th>Cumulative</th>
<th>Slice width (μm)</th>
<th>Tube voltage (kV)</th>
<th>Tube current (mA)</th>
</tr>
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<tbody>
<tr>
<td>80</td>
<td>16</td>
<td>80</td>
<td>50</td>
<td>0.5</td>
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</table>

When X-ray CT apparatus for experimental animals does not introduce a contrast medium, the whole area of the brain appears black and the condition of the brain detail cannot be confirmed. However, we confirmed that there were individuals in the brain of the HIE model rats that had been prepared, with sites of white appearance high in X-ray absorption. In this paper, we refer to the site of white appearance with a high X-ray absorption amount confirmed in the brain of HIE model rat as "high density area". Fig. 5 shows individuals whose high density area was not confirmed. Fig. 6 shows individuals confirmed to have a high density area.

As a result of pathological autopsy on individuals confirmed high density area, we confirmed cerebral edema and cerebral atrophy caused by moisture replacement of brain parenchyma, which is a symptom of hypoxic encephalopathy in all individuals. Fig. 7-8 shows the brain of the rat removed.

![CT images of individuals without high density area](image1)

![CT images of Individuals with high density area](image2)

![CT imaging range](image3)

![Left brain hemisphere atrophy](image4)
As a result of brain removal from Fig. 5, this individual shows that brain atrophy occurs in the left brain.

The confirmed brain atrophy is a state in which no deficiency of the brain parenchyma is observed. Therefore, the brain atrophy may reduce the volume of the brain.

As a result of brain removal from Fig. 6, this individual shows that cerebral edema occurs in the left brain. Moisture is stored in cerebral edema, and brain parenchyma is defective.

8.1.1 Method of examining effects using conventional CT

We examined the effects of plasma irradiated physiological saline and umbilical cord blood comosite using conventional CT. We examined the effect by comparing the compound administered group, the untreated control group, and the control group administered with a mixture of untreated saline and umbilical cord blood.

We induced anesthesia at 4 weeks of age and then evaluated individuals who prepared 1 week old model rats using CT in the same way as shown in Section 7.1.

Using these methods, we evaluated the experimental group and two control groups.

8.1.2 Result of examination of effect using conventional CT

We refer to individuals who received a mixture of untreated saline and umbilical cord blood as Dilution administration experiment. We also refer to individuals who received a mixture of plasma irradiated physiological saline and umbilical cord blood as Compound administration experiment. Table 3 shows the results of evaluating the appearance rate of the high density area using conventional CT.

We compared the control group with the group to which plasma and umbilical cord blood was administered and the group to which umbilical cord blood was administered diluted. As a result, we confirmed that the appearance rate of the high density area is lowered by administration. Furthermore, we confirmed that among the treatment groups, the group with plasma and umbilical cord blood combined administration had the lowest appearance rate of the highest density area.

Table 3 The results of evaluating the appearance rate of the high density area using conventional CT:

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<thead>
<tr>
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<th>Emergence rate of high density area %</th>
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<tbody>
<tr>
<td></td>
<td>Present</td>
</tr>
<tr>
<td>Control (N=16)</td>
<td>81</td>
</tr>
<tr>
<td>Dilution (N=10)</td>
<td>70</td>
</tr>
<tr>
<td>Compound (N=10)</td>
<td>60</td>
</tr>
</tbody>
</table>

Fig. 9 The results of evaluating the appearance of HIE symptoms to the brain by brain removal.
8.2 Appearance evaluation method by pathologic anatomy

When using the method in Section Dilution administration experiment, cerebral edema and cerebral atrophy occur in the brain where high density area is confirmed. Cerebral edema and cerebral atrophy can be reliably evaluated only by appearance because symptoms appear in the appearance of the brain. Therefore, also in this experiment, we performed pathologic dissection for individuals confirmed high density area and evaluated. We performed perfusion fixation after induction of anesthesia, then removed the brain and examined the appearance of the brain to perform pathologic dissection.

8.2.1 Appearance evaluation results by pathologic dissection

Pathological dissection was performed in individuals whose high density area was confirmed by the evaluation of section Dilution administration experiment. Fig.9 show the results of evaluating the appearance of HIE symptoms to the brain by brain removal.

From the table, we found abnormality in all individuals in the control group in individuals with high density area, that is, the control group always had either cerebral edema or cerebral atrophy, which is a symptom of hypoxic encephalopathy. However, we confirmed 43% of the Dilution administration experiment treated group and 40% of the Compound administration experiment treated group, who did not change the appearance of the brain even when the high density area was confirmed in the administered group. The incidence of cerebral edema and cerebral atrophy was lower than that of the non-administered group in both Dilution administration experiment administered group Compound administration experiment treated group. In addition, in group Dilution administration experiment, 14% of individuals with brain atrophy and cerebral edema, some of which exhibited white color, were confirmed.

9. Investigation of effect of compound administration of plasma irradiated water and umbilical cord blood

Based on the results using conventional CT, we obtained a result that the appearance rate of the high density area of the group administered umbilical cord blood and plasma treated physiological saline was 31% lower than that of control. We used multiple fetal groups in all experiments. In addition, we use similar model rat production techniques. Therefore, it is unlikely that the result obtained in this study is that there is a difference in the high density area appearance rate due to individual differences. In other words, the result obtained in this study is that improvement of blood flow, positive effect such as protection of blood vessel or newborn of blood vessel occurs due to some influence by administration of the compound. And they effect make the HIE symptoms by hypoxia and ischemic condition are improved. As a result, there is a possibility that the high density area did not appear.

External evaluation of brain by pathologic anatomy showed either cerebral edema or cerebral atrophy in individuals with high density area in the control group. This result has been demonstrated in experiments using 54 samples, and it can be said that HIE model rats with a high density area always develop cerebral edema or brain atrophy if without treatment. However, in this study, in the group administered complex or umbilical cord blood, there were individuals in whitening of the partial brain tissue and no abnormality was confirmed [9]. We believe that whitening of partial brain tissue is brain cell death and is a preliminary stage of cerebral edema and brain atrophy. Also, even in individuals whose abnormalities could not be confirmed by appearance evaluation of the brain by pathologic autopsy, high density area exists. From this, there is a possibility that an abnormality derived from apoptosis occurs in the cell tissue in the brain of the HIE model rat in which a high density area exists. We can not deny the possibility that there is a microscopic change in the brain by HIE at the present time. However, by administering the compound or umbilical cord blood, individuals who have a high density area but no abnormality can be seen to reduce symptoms when compared with untreated control individuals it had been.

Based on the two evaluation results, we judged that HIE symptoms were alleviated by administration of a combination of atmospheric pressure low temperature plasma and umbilical cord blood. In other words, it was suggested that there is an alleviation effect of HIE symptoms due to administration of composite of atmospheric pressure low temperature plasma and umbilical cord blood.

10. Conclusion

In this paper, we expect treatment of brain diseases due to atmospheric pressure low temperature plasma and umbilical cord blood, examined the combined administration method of them, and examined the effect of the compound on HIE. As a result, we could establish the conditions of combined administration method of viable atmospheric pressure low temperature plasma and umbilical cord blood. In addition, we confirmed that symptoms of brain diseases are alleviated by administering combined atmospheric pressure low temperature plasma and umbilical cord blood. In other words, we considered the combination of atmospheric pressure low temperature plasmas and umbilical cord blood, and some positive influences such as protection and newborn of blood vessels occurred by administration. However, it is still unclear whether atmospheric pressure low temperature plasma and umbilical cord blood will improve the hypoxic condition and ischemic condition, which is a factor of HIE, by administering the compound or alleviate the brain damage to the brain by HIE.

In the future study, we will confirm the influence of combining atmospheric pressure low temperature plasma
and umbilical cord blood from the cell line and confirm the mechanism of action.

Experiments using model rats in this paper were conducted in accordance with the animal experiment guidelines after being approved by the ethics committee of Tokyo City University.

References


