Safety Evaluation of Self-assembling Peptide Gel after Intracranial Administration to Rats Using the Open Field Test

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Self-assembling peptides have been developed as clinical materials, which could scaffold to regenerate nerve cells and hemostatic materials in vivo. However, there has not been enough information for their in vivo application. The safety of self-assembling peptides for the application on the brain was examined using behavioral tests for each rat in this study. Self-assembling peptide gel was administered to the surface of the brain at a volume of 20 µL at 1.5%. After 2 months, the open field test and the prepulse inhibition (PPI) test were performed. There were no significant differences between the peptide gel and the control groups in locomotor distances and in %PPIs in the PPI test. The mean values of the percentage of time the rats stayed in the central area of the open field during the first 5 min and instances of center rearing or face washing in the peptide gel group were significantly higher than those in the control. There were amorphous substance in the subarachnoid region, and infiltrations of mononuclear cells were also observed in the self-assembling peptide gel group. Although the meaning of the effects observed in this study was not fully elucidated, the self-assembling gel produced marginal but significant behavioral and histological effects.

Key words self-assembling peptide; central nervous system; brain; rat

Self-assembling peptides have been employed in various tissue engineering studies. They are characterized by a stable β-sheet structure and undergo self-assembly into nanofibers. The nanofibers form interwoven matrices and further form a hydrogel scaffold. The scaffold is used for a cell culture system. As clinical application of self-assembling peptides, it can be used for scaffolds for implanted cells or hemostatic materials. Because self-assembling peptides are chemically synthesized materials, the use of self-assembling peptides as clinical materials can minimize the risks of biological contamination and allergy. Holmes et al. used self-assembling peptide scaffolds for the cultures of rat PC-12 cells and primary cells from 7-d-old mice cerebellum and hippocampus. Extensive neurite outgrowth and active synapse formation of these cells were observed. Self-assembling peptide may have similar beneficial effects of neuronal tissues.

Although self-assembling peptides have been used in neuronal cell culture systems, there has not been enough information for their in vivo application. For amino acids, there have been studies in which amino acids administered to neonatal chicks by intracerebroventricular injection were shown to have affected the central nervous system (see Discussion). It is of interest whether or not the effects of amino acids by direct application to the central nervous system of neonatal chicks are also observed in other experimental animals which had been administered with self-assembling peptide intracranially. This information is useful for the evaluation of safety of the clinical application of self-assembling peptides for the brain. If the safety of self-assembling peptides for clinical local application for the brain is confirmed, they could be used as a scaffold for the regeneration of nerves or homeostatic materials in the brain. If so, the development of self-assembling peptides would contribute to the improvement of therapies and the development of regenerative medicine.

As an animal model that examines neurotoxicity of a substance when it is applied directly onto the surface of the brain, intracranial administration has been used. As an example of a substance being applied directly onto the surface of the cerebrum in previous studies, artificial degradable dura mater was implanted intracranially to rats after cutting out a circular disk of bone with a bone trephine. The chemical substances composed of the dura mater released gradually into cerebrospinal fluid on the surface of cerebrum. At the endpoint of the observation, the rats were examined by behavior tests such as the open field test and prepulse inhibition (PPI) test to evaluate neurotoxicity. If the neurotoxicity of self-assembling peptides during the direct application on the surface of the cerebrum were evaluated, the method of evaluation used in these previous studies would be useful.

In the present study, the neurotoxicity of self-assembling peptide gels was evaluated after the application onto the surface of the cerebrum of rats. The rats administered with self-assembling peptide gels intracranially were compared to the control by the open field test and the PPI test.

MATERIALS AND METHODS

Tested Materials The tested self-assembling gel was PanaceaGel (Menicon Co., Ltd., Kasugai, Aichi, Japan). The major component of PanaceaGel was a peptide composed of four kinds of 13 amino acids. The primary structure of the peptide was RLDRLALRLDLR (R: arginine, L: leucine, D: asparagine, A: alanine). The 1.5% (w/v) product was used for intracranial administration.

Experimental Animals Male Wistar rats (Oriental Yeast, Co., Tokyo, Japan) aged 8 weeks of old were used. The number of each group was as follows, n=7 for the control, n=10 for the peptide gel-administered group. The mean value and standard error of the body weight of each group at the intracranial administration was as follows: 323±6.0 g for the
control and 339.0±12.6 g for the peptide gel group. There was no difference in this initial body weight between the groups.

**Intracranial Administration of Peptide Gel and Observation** The care, surgical operations, and behavioral tests of rats in this study were in accordance with the guidelines established by the Animal Experimentation and Ethics Committee of Kitasato University School of Medicine and were approved by the committee. For the operation, three anesthetics were administered by intraperitoneal administration: Domitor (1.0 mg/mL of hydrochloric medetomine, Nippon Zenyaku Kogyo, Co., Koriyama, Japan) 0.15 mg/kg as hydrochloric medetomine, Midazolam (Sandoz, Tokyo, Japan) 2 mg/kg, Butorphanol tartrate (Meiji Seika Pharma, Co., Tokyo, Japan) 2.5 mg/kg. After the surgical operations, Antisedan (5.0 mg/mL hydrochloric atipamezole) 0.75 mg/kg was administered intraperitoneally as a medetomine antagonist. Under anesthesia, each rat’s head was immobilized with a stereotaxic instrument (SR-6R; Narishige, Tokyo, Japan). A special auxiliary bar with a round tip was used to secure fixation of the rat’s ears. A circular disk of bone 5 mm in diameter was cut from the cranium of each anesthetized rat using a system composed of a drill and micromotor (Osada Success 40M2, Osada Medical, Tokyo, Japan) with a 5 mm in diameter bone trephine bar (BTB-80, Hasegawa Medical, Tokyo, Japan).

For the peptide gel group, 20 μL of 1.5% of the self-assembling gel, PanaceaGel was administered on the surface of the brain. The control group underwent a sham surgical operation, i.e., a trephination without the administration. After this administration, the cranial bone disk was replaced to the skull. The periosteum and the skin were sutured using 6-0 black nylon (Kawano, Ichikawa, Chiba, Japan).

The rats were housed two to a standard cage on 14/10-h light/dark cycle at 22±2°C air temperature and 45% humidity. Each pair of rats was from the same group; however, one pair was a control rat and a rat that underwent the surgical operation. The rats were maintained on commercial rodent chow for 2 months after the administration, the cranial bone disk was replaced to the skull. The periosteum and the skin were sutured using 6-0 black nylon (Kawano, Ichikawa, Chiba, Japan).

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**Open Field Test** After 2 months, the open field test and the PPI tests were carried out on two consecutive days. Each rat was placed in the center of a white square box (width, 1.0 m; height, 0.4 m) for a 30-min observation. The locomotor behaviors were recorded with a video camera for 30 min. The record was analyzed using an analysis program TimeOFCR1 (Obara Medical, Tokyo, Japan). Total locomotor distance and locomotor distance every 5 min were calculated. In addition, to calculate the time the rat stayed in the central part of the open field, the open field was divided into 25 square areas on the analysis program. The times each rat stayed in the center part of any one of the 9 areas and the surrounding 16 areas were calculated, respectively. The percentage of time the rats stayed in the central part during 30 and the first 5 min was calculated.

During the 30-min observation, the following behaviors were also recorded: the time of the first grooming, the number of times of wall rearing (WR), center rearing (CR), face washing (FW), body washing (BW), defecation and urination. After the open field test, the body weight of each rat was determined.

**The PPI Test** The PPI test was performed by a method described in previous studies. A Startle Response System SR-LAB ABS system (San Diego Instruments, San Diego, CA, U.S.A.) was used. It was composed of a startle chamber with a floor equipped with an electric sensor and a speaker mounted 24 cm above the floor for presenting an acoustic noise burst. Each rat was placed in a cylindrical holder in the chamber and allowed to acclimate for 5 min before the test session. In the test session, four types of acoustic stimulation (a startle pulse of a burst of 120 dB (P alone), combined trials of prepulse (PP, a burst of 70, 75, or 80 dB) followed with a 120-dB pulse (PP70&P, PP75&P, PP80&P) were given to each rat in pseudo-random order. The numbers of acoustic stimulations in a test session were 11 for P alone, PP70&P, and PP75&P, 10 for PP80&P; and 10 for no acoustic stimulation. The startle response was measured with the electric sensor. The mean value of responses for the respective stimulations in a session was calculated.

The percent prepulse inhibition (%PPI) of a startle response was calculated using the following formulae.

\[
\%\text{PPI at PP70} = (1 - \frac{\text{PP70&P}}{\text{P alone}})\times 100 \\
\%\text{PPI at PP75} = (1 - \frac{\text{PP75&P}}{\text{P alone}})\times 100 \\
\%\text{PPI at PP80} = (1 - \frac{\text{PP80&P}}{\text{P alone}})\times 100
\]

If a rat did not show any startle responses throughout the acoustic stimulations or no stimulations in a session, the data was removed from the analysis. There was one rat each removed from the analysis from the control and the peptide gel group.

**Pathological Observation for the Brain** After the PPI test, each rat was euthanized, and the brain was excised. The surface of the cerebrum was observed, and three brains for each group were prepared for pathological observation. The sections from sampled brains were stained with hematoxylin and eosin (H&E) and observed under light microscopy.

**Statistical Analyses** The mean value of each index of each group was calculated and compared between the groups by the t test or the Mann–Whitney U test. Statview version 5.02 software (SAS Institute, Cary, NC, U.S.A.) was used for the statistical analyses. The level of significance was set at p<0.05.

**RESULTS**

The mean body weights and standard errors in the groups at the end of observation period were: 532.3±11.0 g for the control group, 534.5±12.6 g for the peptide gel group. There was no significant difference between the groups.

Figure 1 illustrates the results of open field test. Figure 1(a) illustrates the mean values of locomotor distance every 5 min for 30 min in the open field test in the control and the peptide gel group. There was no significant difference between the groups. Figure 1(b) illustrates the mean values of total locomotion distance of the rats for 30 min in the open field test in the control and the peptide gel group. There was also no significant difference between the groups. Figure 1(c) illustrates the mean values of the percentages of the time that rats stayed in the central area of the open field during 30 min of observation time in each group. The value of the gel group was higher.
than that of the control ($p=0.061$). Figure 1(d) illustrates the mean values of the percentages of the time that rats stayed in the central area of the open field during the first 5 min of observation time in each group. The mean value of the gel group was significantly higher than that of the control. Figure 2 illustrates the numbers of instances of typical behaviors of the rats in the open field test. The mean value of the instances of center rearing in the gel group was significantly higher than that in the control. For face washing, the mean value of the gel group (mean $\pm$ standard error (S.E.) was $3.4 \pm 0.3$) significantly higher than that of the control (mean $\pm$ S.E. was $1.6 \pm 0.6$).

The results of the PPI test are summarized in Table 1. There were no significant differences between the groups for any of the prepulses.

For the surface of the cerebrum of excised brains, there were no particular changes in the controls. However, there was a slight change observed in one rat in the control group. For the gel group, there were brain samples on which gel-like substances were remaining in the area where the gel was administered. Necrosis was observed in one rat in the gel group (data not shown).

In Fig. 3, the typical photographs of the brain samples stained with H&E of the groups are shown. There were slight atrophic changes in the molecular layer of the control samples (Fig. 3(a)). Macrophages that engulfed hemosiderin were observed in the part of the subarachnoid space in one rat in the control group. In another control rat, hemorrhage and slight infiltration of monocytes were observed in the subarachnoid space; while at the same time, there was no remarkable change in one sample. Figure 3(b) is a sample of the self-assembling peptide gel group. In the sample, there was amorphous substance in the subarachnoid region. The slight deposition of hemosiderin and infiltration of mononuclear cells were also observed. The infiltration of mononuclear cells was observed in two of three samples.

### DISCUSSION

This study is a part of the safety evaluation of the application of the self-assembling peptide gel developed by the
The objective of this study was to elucidate whether or not an application of the self-assembling peptide gel on the surface of the cerebrum of rats induces the effects on their behaviors. In our previous study to evaluate the safety of the application of artificial dura mater to the surface of the cerebrum, the artificial dura mater was inserted into the cranium via the hole with a diameter of 5 mm. In this study, the self-assembling gel was applied on the surface of the cerebrum of the rat via the 5-mm diameter hole in the rats’ cranium. The control group underwent a sham surgical operation. The rats were maintained for 2 months, and they were evaluated by the open field test and PPI test.

The general condition, including body weight gain, in the peptide gel group was not different from the control. In the behavioral tests, there were no significant differences between the self-assembling peptide gel group and the control group in the locomotor distances or in the %PPIs in the PPI test. However, the mean values of the percentage of the time that the rats stayed in the central area in the open field test in the first 5 min in the self-assembling peptide gel group were significantly higher than those in the control. The mean values of the percentage of the time that the rats stayed in the central area in the 30 min in the self-assembling peptide gel group were tended to be higher than those in the control. Moreover, the mean value of the number of instances of center rearing and face washing during the open field test in the self-assembling peptide gel group were significantly higher than those in the control, although the former is most likely resulted from the longer stay in the central area. Thus, the present results could be interpreted that the peptide gels at brain surface affects emotional states in rats such as the changes in the preference to the central area and the frequency of face washing behaviors in the open field without marked changes in the locomotor and sensory motor function. It is possible that the observed effects were due to the increased physical pressure induced by intracranial administration of the gel. Also, the peptide gel-induced emotional effects might be associated with sedative effects of amino acids. The peptide gels are composed of the peptide, RLDLRLALRLDLR. There have been studies on amino acids administered to neonatal chicks by intracerebroventricular injection affecting the central nervous system. Suenaga et al. administered L-arginine (R) to neonatal chicks by intracerebroventricular injection. The administration of 1.9 μmol of R decreased spontaneous activity and vocalization, which normally occurs during the stress induced by social separation. Yamane et al. administered 0.84 μmol of L-aspartic acid (Asp) and L-asparagine (D) to chicks by intracerebroventricular injection and discovered that Asp and D attenuated the chicks’ vocalization. Asp and D also decreased the time spent in active wakefulness and induced sedation. Kurauchi et al. administered 0.8 μmol of L-alanine (A) to chicks by intracerebroventricular injection. The chicks administered with L-alanine reduced the posture of active wakefulness and increased the posture of sitting motionless with head dropped. Although these studies were for chicks, the administration of amino acids in the central nervous system may also have sedative effects on rats.

In the pathological observation, in addition to the amorphous substance in the subarachnoid region, increased infiltration of mononuclear cells were observed in the peptide gel group. Although it was not analyzed by histological scoring, the infiltration was relatively serious compared to that in the

![Fig. 3. The Cerebrum of the Rats Administered the Self-assembling Peptide Gel Intracranially in the Open Field Test](image-url)
control.

There were several limitations in this study. First of all, we could not use an adequate positive control group. The candidate for the positive control for this study is the group administered high concentration of self-assembling peptide such as 5 or 15%. Unfortunately, such a product was not available, and we could not establish such a group. Also, for the results of the study, the effects by iatrogenic infection due to the surgical procedure could not be excluded. The necrosis on the surface of the cerebrum may be due to the iatrogenic infection from the surgical procedure. In this study, antibiotics were not administered to the rats. As a simulation for an actual surgical operation as in humans, it would be better to administer antibiotics to the rats before and after the operation to prevent infection.

In conclusion, although it was not elucidated, the longer time of staying in the central area of the open field in the first 5 min, and the increased number of instances of center rearing or face washing in the peptide gel group, were observed in the open field test. In addition, slight changes such as increased infiltration of mononuclear cells were observed in the peptide gel group. Although the peptide gels produced any significant effects at least on the locomotor and psychomotor function, further studies are warranted to elucidate the meaning of the effects observed in this study.

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Conflict of Interest Masashi Tsunoda, Chiemi Sugaya, and Yumiko Sugiura have no conflict of interest. Yusuke Nagai and Kotaro Sakaniishi are employees of the Menicon Co., Ltd., Kasugai, Aichi, Japan.

REFERENCES