

Attachment 4: Test of Malodor Reduction to Male Mouse (12 pages)

Test requested by Litanial Bio Science. Co., Ltd

TEST RPORT

**LBS Culture malodor reduction mechanism
using rat intestinal vagal (parasympathetic)
applying the intragastric administration
and
impact study to the activity of the centrifugal nerve branches**

Test #: ANBAS 00336

Date: Aug. 7th, 2013

Made by: ANBAS Co., Ltd

Exam Outline

1. Test Title:

Studies of the mechanism of malodor reduction by LBS Culture administration:
Study of the effect and the influence on the activity of (parasympathetic) centrifugal nerve branch on rat intestine vagus by the LBS Culture intragastric administration.

2. Test Purpose:

According to the experimental results shows LBS culture (LBS extract) reduces the fishy smell of fish in aquarium and reduce human, animal fecal odor (pet, rodent, bird) of things, and increase the appetite and promoting improvement of diarrhea and constipation in humans and animals. In addition, it was known to promote desired enhancements such as the stomach and intestines gastrointestinal motility and digestion and absorption capacity of the vagus (parasympathetic) nerve and food constipation and fecal odor reduction, and improvement of diarrhea (peristalsis).

Therefore in this study, , we selected urethane anesthetized rats to administrate LBS culture in the stomach in order to clarify the mechanism of fecal odor reduction by LBS Culture administration and the measurement of the change in the activity of the (parasympathetic) centrifugal nerve branch bowel vagus.

3. Test Requested by:

Litanial Bio Science. Co.,Ltd
2-26 Kitamachi Shinnobe Beppu-cho Kakogawa City Hyogo-Ken
Japan Zip 675-0121

4. Test Provided by:

ANBAS Co., Ltd
4-12-17 Toyohashi, Kitaku Osaka City, Osaka Japan.

5. Test Conducted by:

Test Director:

Katsuya Nagai (Osaka University Professor Emeritus)

Test Administrators:

Yuko Horii, Yoshiyuki Fujisaki, Yoshiko Misonoo (Electrophysiological measurement)

Risa Fuyuki (Processing and data analysis)

6. Tested Period:

Test begun: July 26th 2013

Test ended: August 6th, 2013

7. Test Substance: LBS Culture stock solution, solvent (Medium before fermentation)

1. Introduction:

We studied energy regulation mechanism with metabolism under the control of the autonomic nervous system in rats.

In that process, we found changes in the environment from inside and/or outside excite sympathetic nerves which dominate adrenal glands, liver, kidney and pancreas and raises the blood pressure and blood sugar level; on the other hand, substances that reduce the neural activity of those organs gained opposite results indicate that lowering blood pressure and blood sugar level.

In addition, the sent stimulation of grapefruit essential oil commonly known to excite sympathetic nervous system which dominates white adipose tissue, brown adipose tissue, adrenal glands, and the kidney and suppresses the parasympathetic nerves which dominate the stomach by raising the blood pressure to promote (heat production) and energy consumption lipolysis, to suppress the appetite resulting the weight reduction.

Whereas, the sent stimulation with lavender essential oil is commonly known to suppresses sympathetic nervous system which dominates white adipose tissue, brown adipose tissue, adrenal glands, and promotes the parasympathetic nerves which dominate the stomach by lowering blood pressure to suppress the (heat production) energy consumption and lipolysis and to enhance the appetite.

In addition, Lactic acid bacteria, *Lactobacillus johnsonii* La1 (NCC533) and *Lactobacillus paracasei* ST11 (NCC2461) are respectively known to,

- 1) Promote and inhibit the activity of adrenal sympathetic centrifugal branch,
- 2) Suppress and promote the activities of the (parasympathetic) nerve centrifugal branch stomach vagus,
- 3) Result to suppress and promote appetite.

Furthermore, we know those regulates the activity of the sympathetic skin artery by increasing the blood flow of the skin increases the coercive humidity of the skin.

Our experimental study results show LBS Culture (LBS extract) reduces fecal odor (pet, rodent, bird) of humans and animals and reduction of the fishy smell of fish in the aquarium, and promotes improvement of diarrhea and constipation in humans and animals.

Furthermore, when the stomach and intestines vagus (parasympathetic) nerve is promoted, we observe constipation and fecal odor reduction, and improvement of diarrhea gastrointestinal motility and digestion absorption capacity (peristalsis) and then trigger the appetite.

Therefore, in order to clarify the mechanism of fecal odor reduction by LBS culture application, we aim to measure changes in (parasympathetic) nervous centrifugal branch activities intestinal vagus using urethane anesthetized rats by intragastric administration of LBS culture.

2. Methods

In the experiment, each male Wistar rats of about 300g body weight (about 9 weeks old) were housed for about one week and more in a homeothermy room of 24 °C (lit until 8:00 to 20:00) under light-dark cycle of 12 hours. The subjects were administrated with urethane anesthesia after fasted for 3 hours the day of the experiment, inserting the cannula in the stomach administration, and lifting intestine vagus the (parasympathetic) nerve centrifugal branch with a sliver electrode, measured the electrical activity of the nerve method described above (6,8). When this instrument measurement became steady (13 o'clock), the LBS Culture solution was administrated to 1ml/300g rat body weight through cannula into the intragastric administration, and measured electrophysiological changes in the neural activity. In addition, as a comparative experiment applied LBS culture to 1ml/300g rat body weight and measured changes in the neural activity at the time of the intragastric administration in a similar manner. In addition, to ensure the airway by inserting a tube into the trachea until the end of measurement from start of surgery and kept to 35.0 ± 0.5 °C body temperature (rat rectal temperature) in thermal insulation equipment. Activity data of intestinal vagus nerve was averaged in firing rate of 5 per second for 5 minutes each (pulse / 5 s) and conveyed as a percentage, which was set to 100% of the value of 5 minutes before administration (0 min value). In addition, it is calculated the mean \pm standard error from the data, test of statistical significance as a group is performed by analysis of variance (ANOVA) with repeated measures, Mann-Whitney U-test was carried out to get statistically significant difference in absolute value between the neural activity of (0 min) intragastric administration before the start.

3. Results

3.1. Study of the effects of LBS Culture by intragastric administration to intestinal vagus for (parasympathetic) nervous activity.

3.1.1. Exploratory study

In order to examine the effective amount of intragastric administration effects, we created undiluted LBS Culture stock solution, 10-times, 100 times, 1,000 times, and 10,000 times diluted with medium solution, and medium solution alone. Examined the change of vagus nerve activity when each subject solution was intragastric administrated to urethane anesthetized rats at the time of 1ml/300g rat body weight to intestine vagus nerve (parasympathetic) (Intestinal vagal nerve activity, intestinal vagal-NA) and measured in each rat.

In Figure 1, Intragastric administration before the start of the measured data (0 minutes) regarded as 100%, and shown the change in intestinal vagal-NA at time goes. When medium solution have the intragastric administration to 1ml/300g rat body weight went as a comparative experiment and shown intestinal vagal-NA gradual declines slowly, For 60 minutes after intragastric administration intestinal vagal-NA was reduced to 81.7% to minimum (Figure 1).

On the other hand, the application of LBS culture stock solution diluted 10,000-fold with medium solution to intragastric administration of 1ml/300g rat body weight, then Intestinal vagal-NA value increased to 115.4% maximum to 25 minutes after administration, thereafter, the stop value of the vicinity, of the 60 minutes after the administration took a value of 109.1% (Figure 1).

When 1,000-fold diluted with medium solution of intragastric administration to 1ml/300g rat body weight, intestinal vagal-NA does change very little, after 25 minutes intestinal vagal-NA value to 105.4% maximum value, 35 minutes after administration their Intestinal vagal-NA value to 91.4% minimum, the measurement period in the other time perched on the value remained the middle of these value (Figure 1).

When LBS culture stock solution 100-fold diluted with medium solution intragastric administration to 1ml/300g rat body weight, intestinal vagal-NA does change little, intestinal vagal-NA value is 84.9% minimum to 35 minutes after administration, 104.7% next highest value in the 45 minutes after the administration. Intestinal vagal-NA value of the measurement in other period stayed in the middle of the value of those. (Figure 1)

When LBS culture stock solution 10-fold diluted with medium solution intragastric administration to 1ml/300g rat body weight, intestinal vagal-NA does change very little. After 5 minutes the intestinal vagal-NA value 99.6%, 60 minutes after administration becomes 105.6% maximum. Intestinal vagal-NA value of the measurement in other period of stayed in the middle of the value of those. (Figure 1)

When LBS culture undiluted stock solution intragastric administration to 1ml/300g rat body weight, intestinal vagal-NA gradually declines, intestinal vagal-NA value fell to 92.7% minimum in the 60 minutes after the administration. (Figure 1)

Exploratory study of the above cases, we know that the LBS Culture stock 10,000-fold diluted with medium solution raises most strongly intestinal vagal-NA value. Therefore, we chose the LBS Culture stock 10,000-fold diluted with medium solution in the following examined confirmatory using of intestinal vagal-NA value using three rats at each examination.

3.1.2. Confirmatory study

FIG 2 shows medium solution alone without LBS culture and medium solution of undiluted LBS culture to 10,000-fold dilution intragastric administration to 1ml/300g rat body weight, the actual measurement data of intestinal vagal-NA, shows in percentage regarded as 100% neuronal activity in intestinal vagal-NA of (0 min) before start intragastric administration. (Figure 3) As a comparative experiment, when the medium solution without LBS culture intragastric administration to 1ml/300g rat body weight, then intestinal vagal-NA is slightly increased to 5 minutes after the administration, and then reached $104.3 \pm 5.4\%$ maximum, and decreases slowly and gradually thereafter, to 55 minutes after administration intestinal vagal-NA value was reduced to $90.3 \pm 5.0\%$ minimum (Figure 3). On the other hand, when you intragastric administration 10,000 fold dilution to ml/300g rat body weight, intestinal vagal-NA value increased to $115.6 \pm 6.8\%$ maximum to 45 minutes after intragastric administration, Thereafter it decreased slightly stopped on the value of the vicinity (Figure 3). The comparative study Intestinal vagal-NA value of intragastric administration after 5 minutes to 60 minutes between 10,000-fold diluted solution administered group of LBS culture stock solution and the medium solution administration group, former group showed significantly higher value ($P < 0.0005$, $F = 57.6$ by ANOVA in repeated measures). Table 1 shows the absolute value of intestinal vagal-NA before administration (0 min) between 10,000-fold diluted solution administered group of LBS culture stock and the medium solution administration group. Although Table 1 shows the absolute value of the intestinal vagal-NA, there was no statistically significant difference by Mann-Whitney U-test in between two groups.

4. Consideration

The above comparative experiments in intragastric administration to urethane-anesthetized rats between medium solution liquid and LBS Culture 10,000-fold diluted stock solution to 1ml/300g rat body weight, the later solution significantly increases intestinal activity of the vagus (parasympathetic) nerve centrifugal branch with intragastric administration. It is well known fact that when intestinal vagal (parasympathetic) nerve is stimulated promotes gastrointestinal motility (peristalsis) and digestion absorption capacity lead to improvement of constipation and fecal odor reduction, and diarrhea (peristalsis). Thus, the study result of LBS Culture 10,000-fold diluted stock solution intragastric administration promotes the intestinal vagal (parasympathetic) nerve increased gastrointestinal motility (peristalsis) and digestion and absorption capacity suggesting that by reducing the fecal odor and cause appetite improvement and constipation or diarrhea.

5. Conclusion

We investigated the effect of LBS Culture to stomach, using urethane anesthetized rats intestine vagus (parasympathetic) nerve activity (intestinal vagal-NA), and obtained intragastric administration of LBS culture is to promote the intestinal vagal (parasympathetic) nerve activity, by reducing fecal odor be increased gastrointestinal motility (peristalsis), and digestion and absorption capacity, that cause appetite and improvement of diarrhea and constipation.

References:

- 1 Nagai K, et al., *Progr. Brain Res.* 111: 253-272, 1996
- 2 Yamano T, et al., *Neurosci. Lett.* 313: 78-82, 2001
- 3 Nijijima A, et al., *Autonom. Neurosci., Basic & Clinical* 97: 99-102,
- 4 2S0h0e2n J, et al. *Neurosci. Lett.* 380: 289-294, 2005
- 5 Tanida M, et al. *Brain Res.* 1058: 44-55, 2005
- 6 Shen J, et al. *Neurosci. Lett.* 383: 188-193, 2005
- 7 Tanida M, et al. *Neurosci. Lett.* 398: 155-160, 2006
- 8 Tanida M, et al., *Neurosci. Lett.* 389: 109-114, 2005
- 9 Yamano T, et al., *Life Sci.* 79: 1963-1967, 2006
- 10 Tanida M, et al. *Current Topics in Nutraceutical Res.* 5:157-164, 2008
- 11 Tanida M, et al. *Obesity Res. Clin. Practice* 2: 159-169, 2008
- 12 Katsuya Nagai , *Intestinal bacteria Journal* 23: 209-216, 2009
- 13 Horii Y, et al., *Skin Research and Technology* 17:75-81, 2011

図1 LBSカルチャーの原液、原液の培地溶液による10倍希釈液、100倍希釈液、1000倍希釈液、10,000倍希釈液および培地溶液の皮膚塗布刺激による副腎交感神経活動 (adrenal sympathetic nerve activity、ASNA) の変化

Figure 1: LBS Culture Stock Solution, 1/10 Medium solution, 1/100 Medium solution, 1/1,000 Medium solution, 1/10,000 Medium solution and Medium solution cutaneous application stimulation resulting changes in the adrenal sympathetic nerve activity (adrenal sympathetic nerve activity, ASNA).

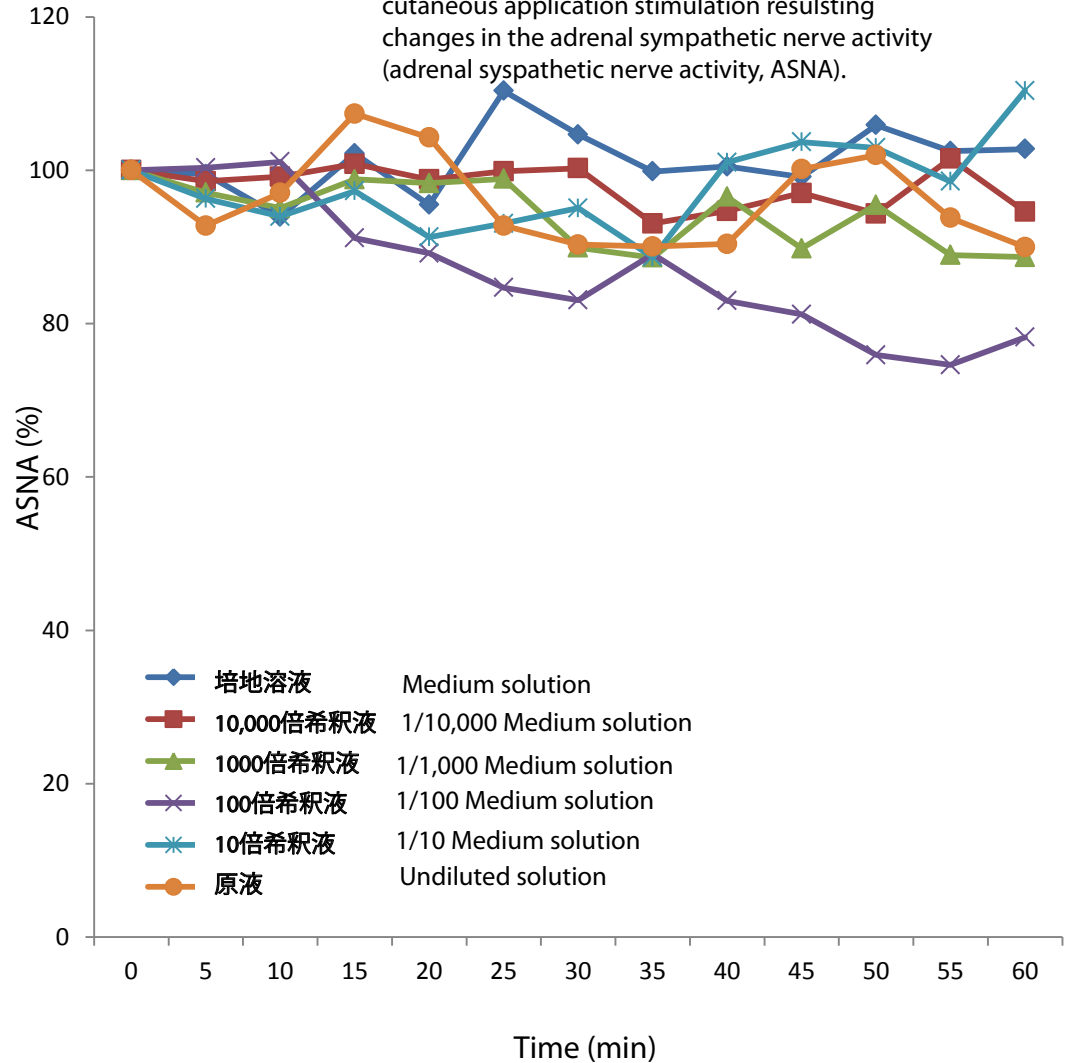
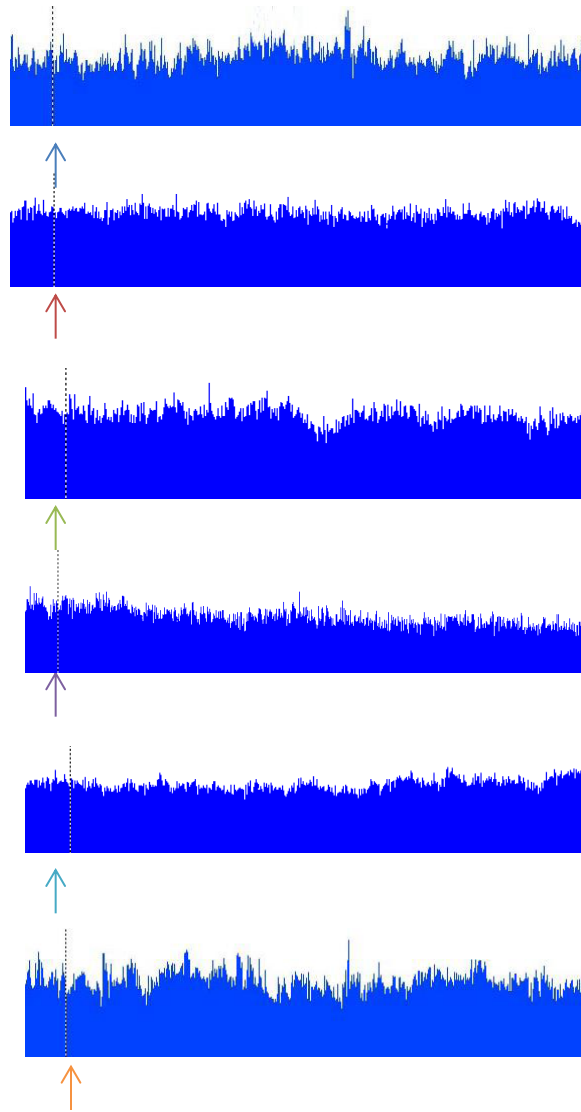


図2 LBS カルチャーの原液の培地溶液による 100倍希釈液および培地溶液の皮膚塗布による副腎交感神経活動 (ASNA) の変化 (実測データ) Figure 2, Medium solution and LBS Culture 100-fold dilution with medium solution cutaneous application to study change of adrenal sympathetic nerve activity (ASNA) (actual data)

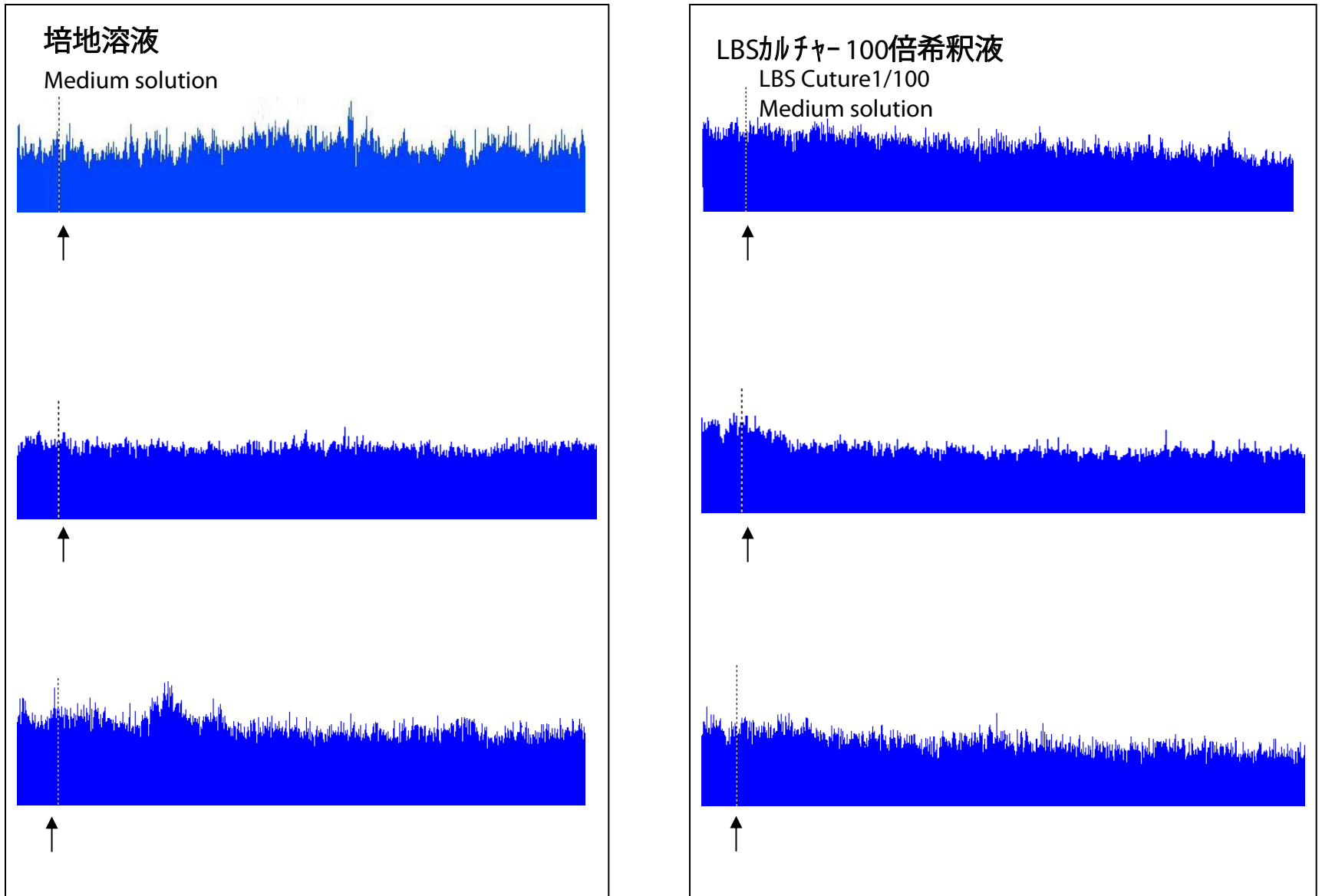


図3 LBSカルチャーの原液の培地溶液による100倍希釈液および培地溶液の皮膚塗布による副腎交感神経活動 (ASNA) の変化

Figure 3, Medium solution and LBS Culture 100-fold dilution with medium solution skin application to study change of adrenal sympathetic nerve activity (ASNA).

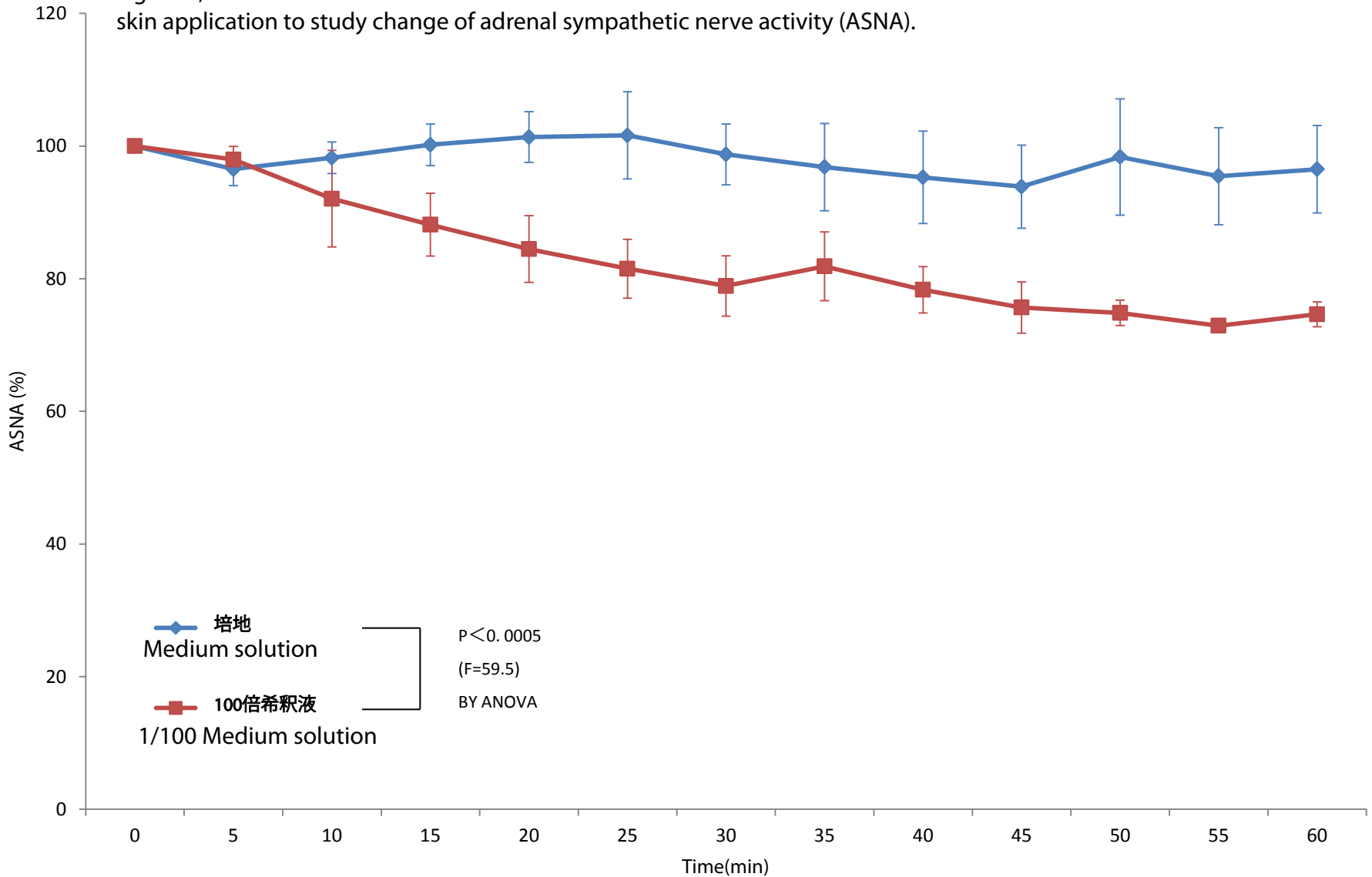


表 1 培地溶液もしくはLBSカルチャー100倍希釈液の皮膚塗布開始直前 (0分)のASNAの絶対値 Table 1, Absolute value of the ASNA of medium solution and LBS Culture 100-fold medium solution immediately before skin application (0 min) .

| 実験群 Experimental group | 神経活動(spikes/5s)(Mean± SEM) Nerve activity |
|---|--|
| 培地溶液 Medium Solution | ASNA(n = 3) 227± 21 |
| LBSカルチャー LBS Culture 1/100 medium solution | 216± 18 |

Mann-Whitney U-testによる統計的有意差なし
No statistically significant difference

図4 LBSカルチャーの原液、原液の培地溶液による10倍希釈液、100倍希釈液、1000倍希釈液、10,000倍希釈液および培地溶液の皮膚塗布刺激による皮膚動脈交感神経活動 (cutaneous arterial sympathetic nerve、CASNA) の変化

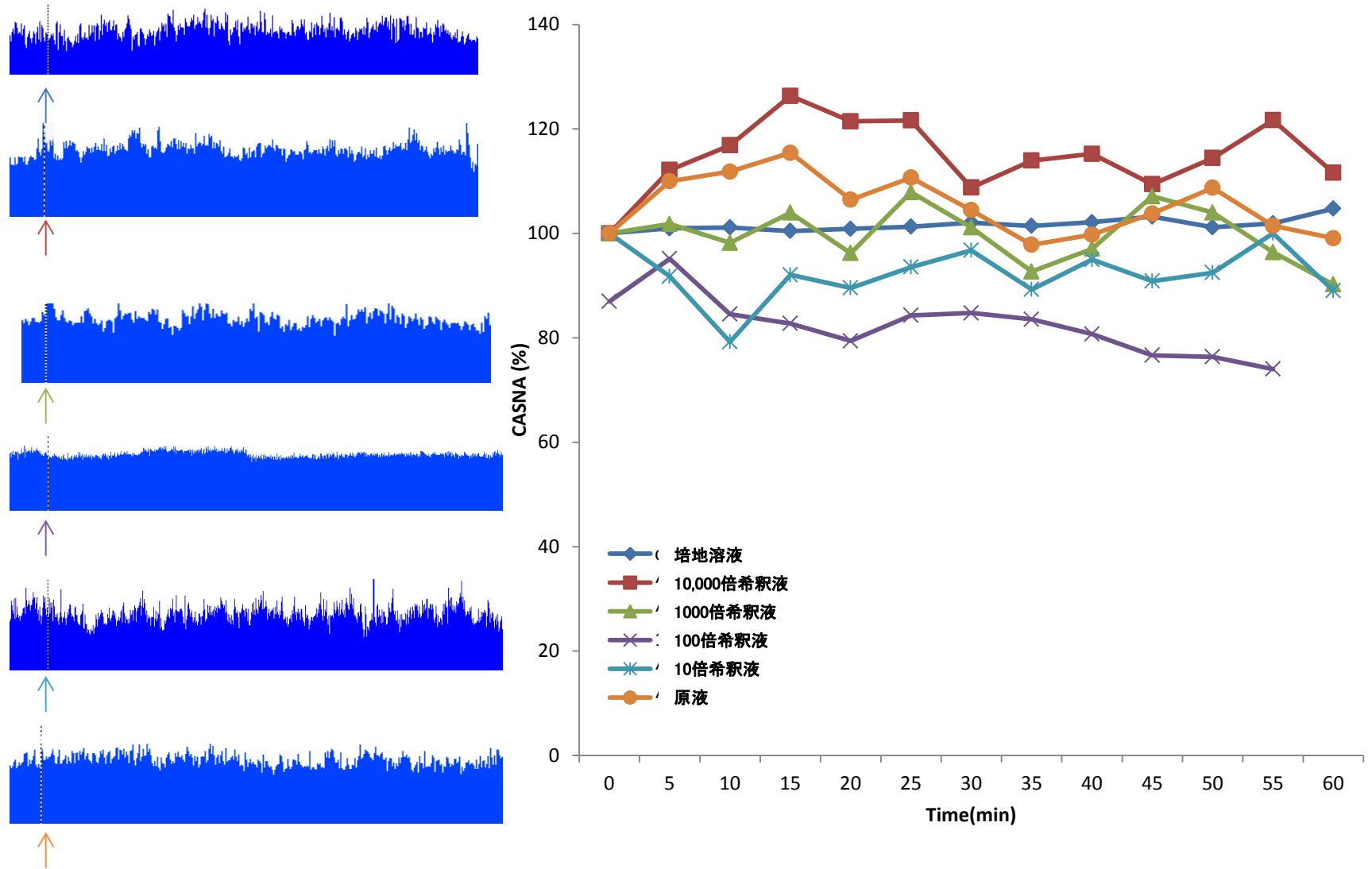


図5 LBSカルチャーの原液の培地溶液による100倍希釈液および培地溶液の皮膚塗布による皮膚動脈交感神経活動(CASNA)の変化(実測データ)

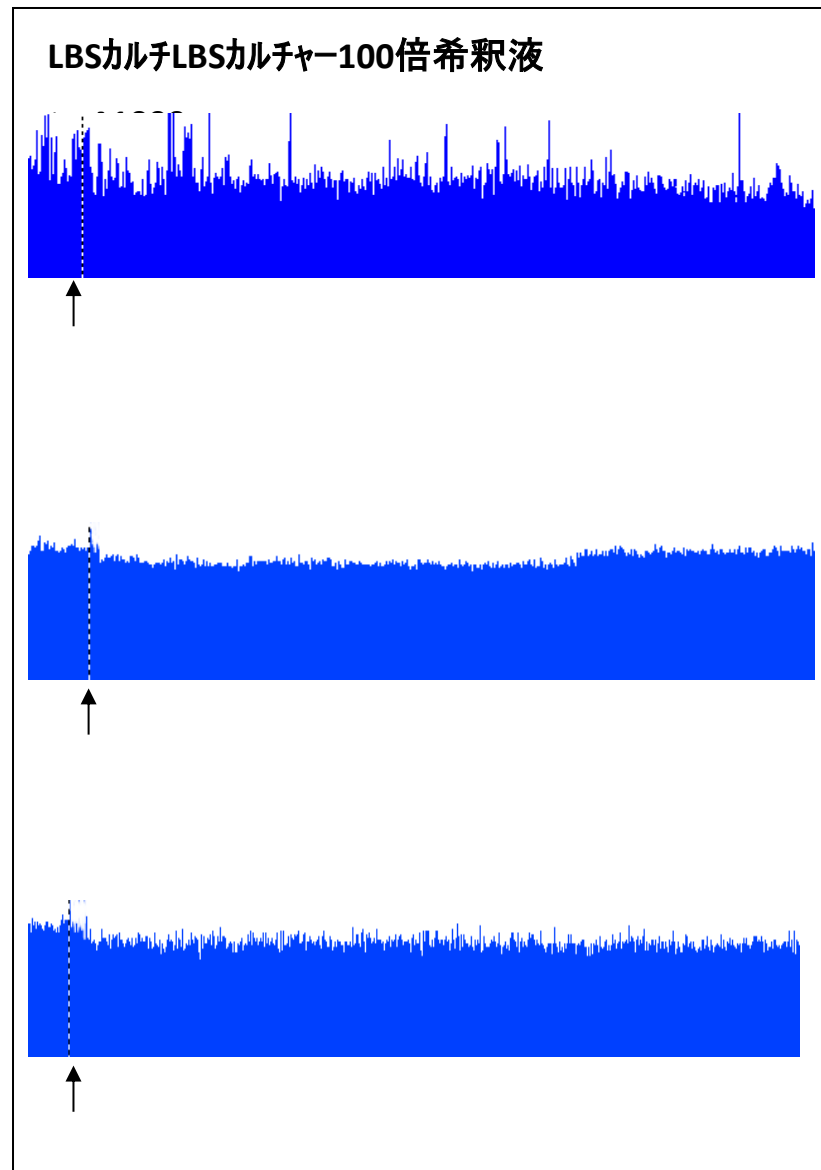
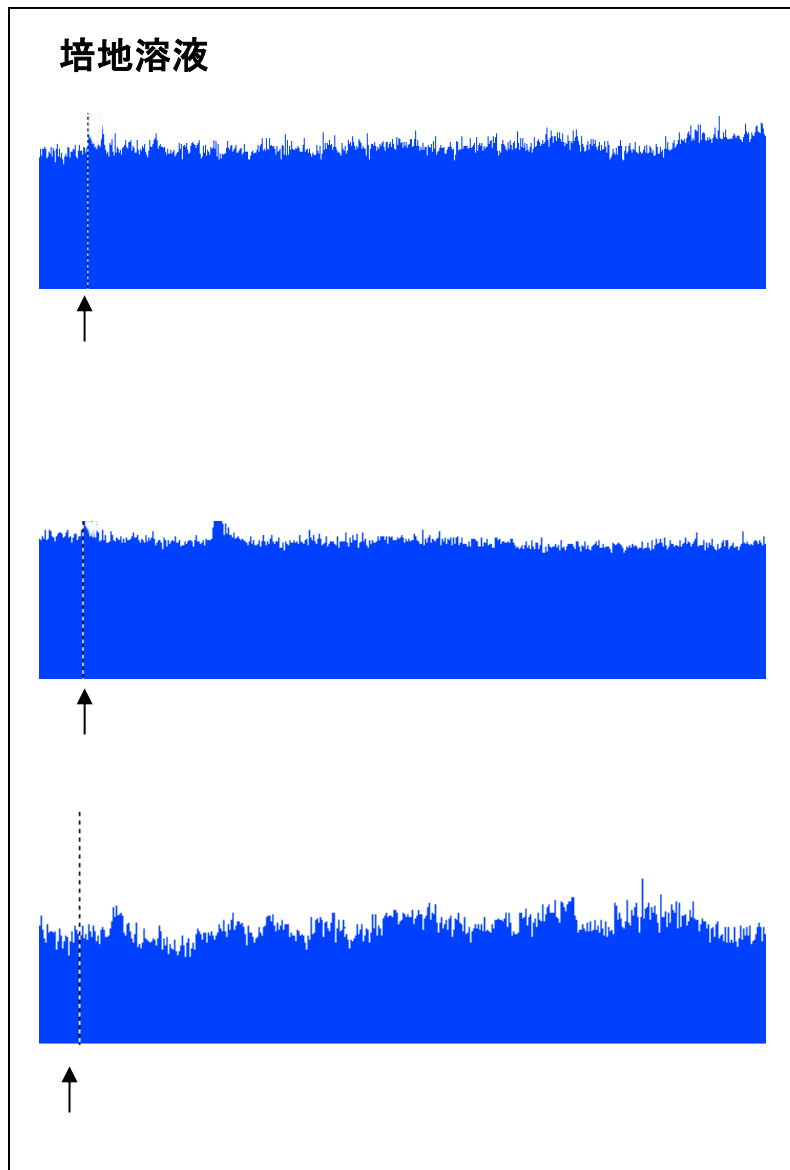


図6 LBSカルチャーの原液の培地溶液による100倍希釈液および培地溶液の皮膚塗布による皮膚動脈交感神経活動(CASNA)の変化

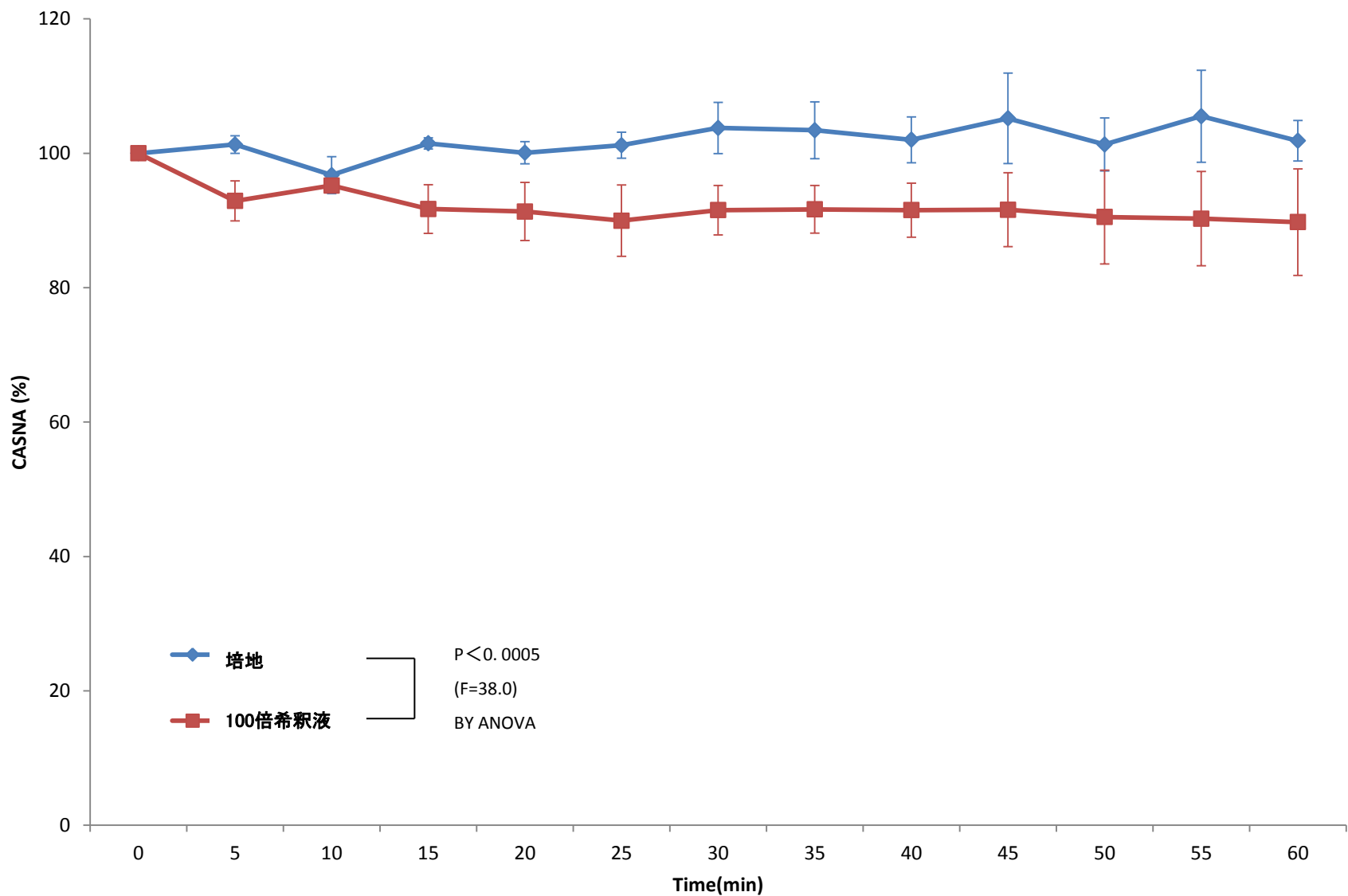


表2 培地溶液もしくはLBSカルチャー100倍希釈液の皮膚塗布開始直前(0分)のCASNAの絶対値

| 実験群 | 神経活動 (spikes/5 s) (Mean ± SEM) |
|----------|--------------------------------|
| | CASNA (n = 3) |
| 培地溶液 | 250 ± 14 |
| LBSカルチャー | 250 ± 37 |

Mann-Whitney U-testによる統計的有意差なし