Development of direct pain evaluation test by using Substance P

Kazuyoshi Tsuchiya[1], Yusri[1,3], Kagemasa Kajiwara[2] and Minoru Kimura[2]
[1]*Department of Precision Engineering, Tokai University, Japan*
[2]*Department of Molecular Life Science, Division of Basic Medical Science and Molecular Medicine, School of Medicine, Tokai University, Japan*
[3]*Faculty of Mechanical Engineering, University of Malaysia Pahang, Malaysia*

tsuchiya@tokai-u.jp

Abstract

In order to design a microneedle, the size of outer diameter is important to reduce the pain. Therefore, we need a pain evaluation test to determine the marginal area for outer diameter size of microneedle for designing a painless microneedle. In this study, we focused on the Substance P (SP), which is a pain transmitter, and SP is also expressed at local inflammation as mediator. Firstly, we injected mouse's skin with various diameter sizes of injection needle. An indirect immunofluorescence test is applied on the tissue skin before the marginal area of outer diameter size to reduce pain is determined by calculating the quantity of SP appearance with image processing. From the results, the amount of SP after stimulated by outer diameter size of over 100 μm is larger than its negative control. It is found that the value which amount of prepared SP areas is divided by surface area of the injected needle is constant at 0.05. From here, we proved that this pain evaluation method is effective to directly evaluate the amount of SP quantitatively.

1 Introduction

A pain evaluation test is important in the design of a painless microneedle since the maximum size of outer diameter within the marginal region of painless can be investigated with it. Here, the pain evaluation test through stress assessment test has been proposed [1]. By measuring the fluctuation in salivary alpha amylase (sAA) levels during the injection, where the sAA is the evaluating factor of stress, it was confirmed that this technique is possible as an objective evaluation of pain and the maximum outer diameter for pain was confirmed between 95 μm and 100 μm. However, this method cannot evaluate pain on local pain region since it was used to

evaluate pain through stress. Therefore, in this study, we focused on the expression of Substance P (SP) [2], which is a pain transmitter. The SP has been proven expressed only at the stimulated region after mouse skin was stimulated with physical stimulus. Thus, we conducted an experiment by injecting mouse skin with various outer diameter sizes of needle and determine the maximum size of outer diameter within the marginal region of painless by measuring the quantity of expressed SP with image processing.

2 Pain Evaluation Test Procedure

Substance P is involved in nociception, transmitting information about tissue damage from peripheral receptors to the central nervous system to be converted to the sensation of pain [3]. The effectiveness of this evaluation method is investigated by observing the differences of behaviour and characteristics on the expression of SP between stimulated groups and a control group. Here, genes in the mouse and human genomes are 99% conserved [4]. Therefore, mice frequently have syndromes similar to human because of their close metabolic and internal anatomical similarities to human beings, and mice have similarities in both physical behaviour such as a size of cell and biological behaviour such as a response to the stimulus.

SP which expressed on the tissue after the needle stimulation is acting as an antigen. Then, a primary antibody, which is IgG from other mice, was applied onto tissue section for binding with the SP. Next, by applying a secondary antibody (Alexa Fluor 594), it will react and recognize only the binding of SP and IgG. After the reaction, by applying excitation light of 590 nm from fluorescence microscope on the tissue section, fluorescence signal of SP is appeared to be red in colour. In order to evaluate and measure the expression of SP quantitatively, image process software, ImageJ was used.

Table1shows the type of injection needles which were used as physical stimulus on mice skin. A substitute needle was being used in this experiment since it has been proved that there is no significant difference for the tip and surface on injection needle with size of outer diameter below than 200 μm by the sAA level test [1]. The substitute needle was not a commercial needle, where the bevel angle was set to the standard bevel, which is 12° (sharpest of the bevel types for blood collecting needle) but silicone coating and lancet were not applied on the needle tip.

The experiment was carried out with 20 times of injection in the range of 4 mm^2, without repetition on the same injection spot with a depth of 3 mm within 1 sec. Then, tissue fixation was applied. Tissue section with thickness of 2 µm was made from paraffin block. After that, paraffin was removed from these paraffin embedded tissue sections through xylenes and graded alcohol series (ethanol) and rinsed for 5 min in PBS.

Table1: Type of injection needle.

	Substitute Needle	Nanopass 33	30 G	27 G
Outer Diameter [µm]	100	200	300	400
Bevel angle [°]	12	12	12	12

3 Results and Discussions

In this study, area of fluorescence signal of red in each pixel was taken as an evaluation value in order to measure the expression of SP quantitatively. All of the images which observed on the cross section of tissue were taken from fluorescence microscope and processed in 640 × 640 pixels via ImageJ. Next, these colour images were converted into grayscale and processed within the range of 0 to 255. Since the negative control was 23±2.28, the measurement was set to be calculated from the value of 25 to 255. Next, measurement scale was set to 0.690 [pixels/μm^2] in order to calculate the area of the expression of SP after injected with 100 µm, 200 µm, 300 µm and 400 µm of outer diameter of needle. After that, the quantity of SP was compared so that the SP can be evaluated quantitatively. The results were shown in Fig.1, where the contact area indicated the surface area of needle including area of bevel part and cylinder part of needle of the needle, which contacted to the tissue skin. The area, which consists of SP, after injected with 200 µm of outer diameter was 87326.173 μm^2, after injected with 300 µm of outer diameter was 122959.41 μm^2 and after injected with 400 µm of outer diameter was 138143.21 μm^2. However, the SP was confirmed not existed after the comparison between image of 100 µm of outer diameter and negative control [1]. From Fig.1, between diameters of 200 µm, 300 µm and 400 µm, the correlation was positive with 0.974 between outer diameter of injection needle and the quantity of the expression of SP. Therefore, when the outer diameter is larger than 200 µm, as the outer diameter keeps increase, the amount of

the expression of SP will also increase. Thus, it was possible to evaluate the expression of SP quantitatively through this method. The results shows the same phenomenon as the phenomenon where the marginal maximum outer diameter for pain was confirmed between 95 μm and 100 μm by using the sAA level test [1].

It was found that the value after divided the amount of SP areas by contact area was constant at 0.05. From here, it was confirmed that the direct pain evaluation method is effective to evaluate the amount of SP quantitatively.

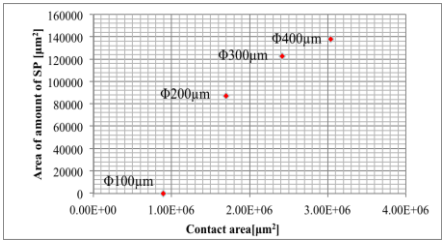

Figure 1: Comparison between contact area of needles and area of amount of SP.

4 Conclusions

In this study, by evaluating the expression of SP quantitatively after injected with various size of outer diameter needle and compared with negative control, the following knowledge were obtained: The expression of SP was confirmed at surrounding area of damaged tissue only after injected with injection needle which has outer diameter larger than 200 μm. Amount of SP is possible to be calculated quantitatively by image processing. The changing on the amount of SP was confirmed by the changing of outer diameter size.

References:

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