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Analgesic effects of chitin and chitosan

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Abstract

The analgesic effects of chitin and chitosan on inflammatory pain were evaluated using the acetic-acid-induced writhing test in mice. When chitin and chitosan suspensions were mixed with the 0.5% acetic acid solution (chitin-AC and chitosan-AC, respectively) and administered intraperitoneally in mice, both agents induced a dose-dependent decrease in the number of the abnormal behaviors (writhing) due to pain, including extension of the hind legs, abdominal rigidity, and abdominal torsion. This effect was greater in the animals administered the chitosan-AC than in those administered the chitin-AC. In vitro study indicated that addition of the chitin or chitosan suspension increased the pH of the AC, and that this effect was greater in the chitosan than the chitin. Furthermore, the level of bradykinin in the peritoneal lavage fluid in the animals administered the chitin-AC was lower than in the animals administered the chitosan-AC. In vitro study showed that the chitin particles absorbed bradykinin more extensively than the chitosan particles. These results suggest that the main analgesic effect of chitosan is the absorption of proton ions released in the inflammatory site, while that of chitin is the absorption of bradykinin.

Keywords: Analgesia; Chitin/chitosan; Writhing test; Mice

1. Introduction

Chitin and chitosan are found to induce granulation tissue with angiogenesis and less scar formation (Minami, Okamoto, Tanioka, Sashiwa, Saimoto, Matsuhashi et al., 1993; Okamoto, Minami, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1993a; Okamoto, Minami, Matsuhashi, Sashiwa, Saimoto, Shigemasa et al., 1993b; Okamoto, Shibazaki, Minami, Matsuhashi, Tanioka et al., 1995; Okamoto, Southwood, Stashak, Norrdin, Nelson, Minami et al., 1997. In addition, some investigators reported that chitin and chitosan induce analgesia. Allan, Altman, Bensinger, Ghosh, Hirabayasi & Neogi (1984) found that chitosan provided a cool and pleasant soothing effect when applied to open wounds. Ohshima, Nishino, Yonekura, Kishimoto & Wakabayashi (1987) reported that chitin proved excellent pain relief in 83 out of 91 cases who received the agent topically over open wounds such as burns, skin abrasions, skin ulcers, skin graft areas, and so on. We have also experienced that animals did not feel pain when their wound were covered with chitin and chitosan (Minami et al., 1993; Okamoto et al., 1993b). These reports describe only the clinical effects of chitin and chitosan on pain relief. To our knowledge, no one has yet investigated the underlying mechanism of the effect. In this paper, we experimentally evaluated the analgesic effects of chitin and chitosan using the acetic-acid-induced writhing test in mice (Nakamura & Shimizu, 1981), a technique widely used for the development of analgesic drugs.

2. Materials and methods

2.1. Animals

Sixty-four mice (female, 5–8 weeks) were used in this study. The animals were purchased from Curea Laboratory Co., Ltd (Japan).

2.2. Reagents

Chitin/chitosan: Chitin and chitosan were supplied by Sunfive Co., Ltd (Japan). Chitin (MW: 300 kD, derived from squid pen) and chitosan (MW: 80 kD, derived from crab shell chitin) with a mean particle size of 3.5 μm were each sterilized using ethylene oxide and suspended in sterile saline at a concentration of 10 mg/ml. These agents showed <10 and >80% deacetylation, respectively. The molecular weight and deacetylation were determined by the viscosity method (Shigemasa, Matsuura, Sashiwa & Saimoto, 1996

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Table 1
Effects of chitin and chitosan on number of writhing. (*Significant difference compared to 0.5% acetic acid ($p < 0.05$)

Agents	Concentration (mg/ml)	Number of Writhing	Writhing depression rate[a] (%)
0.5 % Acetic acid	–	29.7 ± 1.5	(0)
Chitin	1	30.7 ± 1.2[b]	(0)
	5	24.0 ± 1.0*	(19.1)
	10	19.0 ± 1.0*	(36.0)
Chitosan	1	23.7 ± 3.5*	(20.2)
	5	19.7 ± 1.5*	(33.7)
	10	13.0 ± 1.0*	(56.2)

[a] Writhing depression rate $= (B - A)/B \times 100$. *A*: Writhing time during the 10 min period from 10 to 20 min after the intraperitoneal chitin- or chitosan-AC administration. *B*: Writhing during the 10 min period from 10 to 20 min after 0.5% AC administration.
[b] Mean ± SD ($n = 4$).

and IR method (Tokura & Nishi, 1995), respectively. Both chitin and chitosan suspensions were adjusted with sterile saline to final concentrations of 1, 5, and 10 mg/ml before use.

Acetic acid (AC): 99.5% acetic acid (Nakarai Co. Ltd, Japan) was diluted to 0.1, 0.5, 1.0% with sterile saline and then passed through a 0.45 μm pore-sized filter.

Bradykinin: Bradykinin (Wako Pure Chemical, Japan) was adjusted to 1 μg/ml with sterile saline.

2.3. Writhing test

The writhing test using AC was performed by the method of Nakamura and Shimizu (1981). The optimum time and AC concentration for the writhing test were investigated in a preliminary study. AC mixture were administered to the mice intraperitoneally at a volume of 25 μl/g body weight at AC concentrations of 0.1, 0.5, and 1.0%. After the injection, the behavior of the mice was recorded with a video camera for 60 min. At for every 10 min period, the extent of three types of abnormal behavior (writhing) due to the pain, i.e. extension of the hind legs, abdominal rigidity, and abdominal torsion, was quantitated (number of extensions, number of contractions, number of abdominal twists). In the second period, 10 min ranging from 10 to 20 min after the injection, the pain peaked in the 0.1 and 0.5% AC groups. The number of writhings in the 0.1% group was one third of that in the 0.5% group. In the 1.0% AC group, some mice died the day after the injection. As a result, optimum concentration was determined to be 0.5% AC, and the 10 min period from 10 to 20 min after injection was determined to be suitable for the test.

2.4. Effects of chitin and chitosan on AC-induced writhing test

Chitin/chitosan suspensions at concentrations of 1, 5, 10 mg/ml were mixed with same volume of 0.5% AC. The mixture of AC and chitin suspension at the concentration of 1 mg/ml was named the '1 mg chitin-AC solution', and the remaining mixtures were named in a similar manner. Each mixture was administered to the mice intraperitoneally at a volume of 25 μl/g body weight ($n = 4$). For the control, the mixture of saline and 0.5% AC was injected. Changes in the writhing pattern due to chitin/chitosan were calculated as follows.

Writhing depression rate $= (B - A)/B \times 100$. *A*: Writhing time during the 10 min period from 10 to 20 min after the intraperitoneal chitin- or chitosan-AC administration. *B*: Writhing time during the10 min period ranging from 10 to 20 min after 0.5% AC administration.

2.5. Relationship between chitin/chitosan and proton ion levels

The pH levels of 1, 5, 10 mg chitin- and chitosan-AC were measured with a pH meter (Horiba, Japan).

2.6. Effect of chitin/chitosan on bradykinin production

25 μl/g body weight of each mixture was administered intraperitoneally in mice ($n = 4$). For the control, a mixture of saline and 0.5% AC was injected. In addition, the pH of the AC was adjusted to the same level in each chitin- and chitosan-AC solution, and then the pH-adjusted solutions were administered intraperitoneally in mice ($n = 4$). After 20 min, the mice were euthanized by decapitation under ether anesthesia. 5 ml of saline was administered intraperitoneally, the abdomen was massaged gently with the fingers for 1 min and opened, and then the fluid in the abdomen was harvested. The fluid was diluted to 10 times with saline, and then passed through a 0.45 μm pore-sized filter. The levels of bradykinin in the diluted fluids were measured with a Bradykinin EIA Kit (Dainippon Seiyaku, Japan).

2.7. Absorption of bradykinin with chitin/chitosan in vitro

One ml volume of chitin/chitosan suspensions (10 and 100 mg/ml) were transferred into tubes, respectively. 4 ml of bradykinin solution (1 μg/ml) was added to each tube, and then each tube was shaken once every 5 min for 60 min at room temperature. After shaking, the tubes were

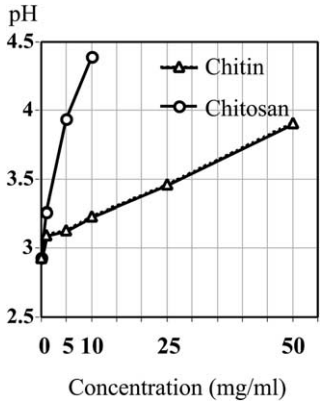

Fig. 1. Relationship between chitin/chitosan and proton level.

centrifuged ($850 \times g$, 10 min), and the supernatants were harvested and passed through a 0.45 μm pore-sized filter. Levels of bradykinin in the supernatants were measured by the above method. For the control, a mixture of 4 ml of bradykinin solution and 1 ml of saline was prepared and a similar procedure was performed.

2.8. Statistical analysis

ANOVA with Scheffe's F test was carried out with STATVIEW-IV program using a Macintosh computer. Difference at a probability of $P < 0.05$ were considered significant.

3. Results

3.1. Effects of chitin and chitosan on the writhing time

The effects of chitin and chitosan on the writhing time are shown in Table 1. The writhing time was decreased significantly in the 5 and 10 mg chitin-AC groups, and in all of the chitosan-AC groups. The writhing depression rates of 5 and 10 mg chitin-AC were similar to those of 1 and 5 mg chitosan-AC, respectively.

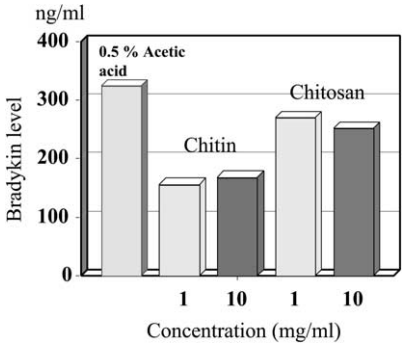

Fig. 2. Effects of chitin/chitosan on bradykinin production in vivo.

3.2. Relationship between chitin/chitosan and the proton level

Fig. 1 shows the relationship between chitin/chitosan and the proton ion level. The pH in the solution increased as the concentrations of chitin and chitosan increased, but the rate of pH increase was slower in the chitin-AC group than in the chitosan-AC group. The pH levels of the 10, 25, and 50 mg chitin-AC solutions were similar to those of 1, 2.5, and 5 mg chitosan-AC solutions, respectively.

3.3. Effect of chitin/chitosan on bradykinin production in vivo

Fig. 2 shows the effect of chitin/chitosan on bradykinin production. Bradykinin levels in the 1 and 10 mg chitosan-AC solutions were decreased slightly (16.7 and 22.2% decreases, respectively) compared with the control (AC alone). On the other hand, bradykinin levels in the 1 and 10 mg chitin-AC solution were decreased greatly (51.9 and 48.1% decreases, respectively) compared with those in the chitosan-AC solution. With pH adjustment, there was a change in the level of bradykinin in the chitin group, whereas no such change was seen in the chitosan group.

3.4. Absorption of bradykinin with chitin/chitosan in vitro

The absorption of bradykinin with chitin/chitosan in vitro is shown in Table 2. Ten and 100 mg/ml of chitin absorbed 54.8 and 54.8% of bradykinin, respectively. 10 and 100 mg/ml of chitosan absorbed 19.4 and 16.1%, respectively.

4. Disccusion

Chitin and chitosan have been found to reduce the inflammatory pain due to intraperitoneal administration of acetic acid (AC). This effect was also been shown to be dose-dependent. These discoveries now call for experimental proof that chitin and chitosan have potent analgesic actions. In a preliminary study, AC was found to induce pain. However, when sodium acetic acid was administered to test animals intraperitoneally, no abnormal behavior (writhing) due to pain developed (data not shown). These data indicate that the writhing is influenced greatly by the proton ions released from the AC, i.e. the pH of the AC. When the chitosan suspension was mixed with AC, the chitosan particles combined chemically with the proton ions in the AC solution, the amino group in the position of C2 changed into NH_3^+, and subsequently the particles resolved in the solution. Therefore, the decrease of the writhing time due to chitosan is strongly related to the absorption of the proton ions. An in vitro showing increase in the pH of AC by the addition of chitin as well as chitosan, provides further evidence that chitin also absorb slightly the proton ion, though chitin does not resolve in acetic acid solution. When the concentration-dependent changes in the pH of

Table 2
Absorption of bradykinin with chitin/chitosan in vitro

Agents	Concentration (mg/ml)	Bradykinin (ng/ml)	(%)
Saline	–	37.2[a]	(100)
Chitin	10	16.8	(45.2)
	100	16.8	(45.2)
Chitosan	10	30.0	(80.6)
	100	31.2	(83.9)

[a] Mean ($n = 2$).

the chitin- and chitosan-AC solutions are compared with the writhing depression rate, the chitin-AC solution reached the same pH as the chitosan-AC solution only at a ten-fold greater concentration. It is speculated that the difference of pH between the chitin- and chitosan-AC solutions is based on degree of deacetylation, in other words, the number of amino groups in the structure of the molecule. If the writhing depression is based on only absorption of the proton ion, the concentration of the chitin-AC solution must be 10 times higher than that of the chitosan-AC solution to obtain the same writhing depression rate of the chitosan-AC. Actually, the present result showed that the chitin-AC solution induced a similar writhing time to the chitosan-AC solution when its concentration was only at 2–5 times higher. From this result, it is speculated that another mechanism contributes to the chitin-induced depression of writhing.

Bradykinin is one of the main substances related to pain. In the present study, the levels of bradykinin induced by the chitin- and chitosan-AC solution in the peritoneal lavage fluid were lower than that of the 0.5% AC. Regarding the chitosan-AC solution, the decrease of the bradykinin level is thought to stem from the decrease of tissue injury by the AC following the absorption of the proton ions. On the other hand, since the chitin particles absorb fewer proton ions than the chitosan particles, the decrease of the bradykinin level by the chitin-AC solution suggested that there is another cause without absorption of proton ion. In vitro study showed that the chitin particles absorbed bradykinin more extensively than the chitosan particles. From these results, we can conclude that the absorption of bradykinin may be one of the main analgesic effects of chitin. In the present study, we could not explain by which modality bradykinin is adsorbed by chitin particle. Further investigation will uncover how chitin adsorbs bradykinin.

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