Inhibitory Effects of Valproic Acid in Oxaliplatin-Induced Neuropathy in Rat Model

2018

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Contents

1. ABSTRACT

AIM: Oxaliplatin is a third-generation platinum-based chemotherapy drug, introduced for management of the advanced stages of metastatic colorectal cancer. However, repeated administration of oxaliplatin induced acute and chronic peripheral neuropathy. Valproic acid (VPA) is a neurotherapeutic drug used widespread and worldwide as therapy for seizures, bipolar disorder, and migraine, including children, adult and women of reproductive age.

MATERIALS AND METHODS: In the present study, we investigated the effect of VPA in prevention of oxaliplatin-induced periphery neuropathy in the rat model. We demonstrated that VPA (300 mg/kg) relieved the oxaliplatin (4mg/kg)-induced peripheral neuropathy using behavioral tests, biochemical tests, and histopathological and immunohistochemical evaluations.

RESULTS: VPA administration significantly attenuated the mechanical hyperalgesia by oxaliplatin-induced in rats. VPA exerted a significant

protective effect by reducing the occurrence of multinucleolated neurons and the nucleolar eccentricity caused on lumbar dorsal root ganglion from oxaliplatin-treated rats. It revealed an inhibitory effect of VPA on the number and activation of microglia and astrocytes in the dorsal horn of the spinal cord. However, VPA was unable to prevent demyelination and degeneration of nerve fibers from oxaliplatin-induced peripheral neurotoxicity.

COLUSION: The present results demonstrated for the first time that VPA administration ameliorated the oxaliplatin-induced behavioral, biochemical and histopathological changes in rats. The VPA-mediated effects in this study may be attributed to neuroprotection properties and ameliorating oxaliplatin-induced astrocytes and microglial activation. VPA may offer a dual protective approach against etiological factors and resulting maladaptative plasticity.

Keywords: Oxaliplatin, Peripheral neuropathy, Mechanical allodynia, Valproic acid, Astrocyte and Microglia, Nucleolar eccentricity

2. INTRODUCTION

Oxaliplatin is a third-generation platinum-based chemotherapy drug in various solid tumours, in particular, it was introduced for the management of the advanced stages of metastatic colorectal cancer [1]. However, it has been reported that repeated administration of oxaliplatin induced severe acute and chronic peripheral neuropathy [2]. Oxaliplatin induced neuropathy can persist from months to years beyond chemotherapy completion, causing significant challenges for cancer survivors due to negative influence on function and quality of life. Oxaliplatin neurotoxicity resulted in chemotherapy dose reductions or early discontinuation [3].

Acute neuropathy shows cold hyperalgesia in the early phase and includes acral paresthesias enhanced by exposure to cold in 71 to 95% of all patients [4,5]. It has been thought that the acute neuropathy is not due to morphological damage of the nerve [6] and is due to alternations of voltage-gated Ca2+ and K+ channels [7]. On the other hand, the dose-limiting toxicity of this compound is the development

of peripheral neuropathy with glove-and-stocking distribution sensory loss, combined with paresthesia, dysesthesia, pain, and motor neuropathy [3,6,8]. A chronic neurological syndrome, related to the total cumulative dose as well as the dose-intensity of treatment persists between and after treatments [9] negatively influencing patient's quality of life. Thus, these neuropathies are a major clinical problem in oxaliplatin chemotherapy.

To ameliorate oxaliplatin-induced neuropathy, various treatments by animal experiments have been suggested including gabapentin [10], neurotropin [6], carbamazepine [11], phosphatidylcholine [12], N-palmitoylethanolamine [3], exenatide [8], and goshajinkigan [2]. There is no currently univocally-accepted proven therapy for oxaliplatin-induced neuropathy. Most randomized controlled trials testing a variety of drugs with diverse mechanisms of action failed to reveal an effective treatment. Recently, most reports including glutathione [13], duloxetine [14], Vitamin E [15], oxycodone [16], goshajinkigan [17], pregabalin [18] and MR309 (a novel selective sigma-1 receptor ligand previously developed as E-52862) [19] show

some effects. Neuroprotective, safe, preventive agents as adjuvant to chemotherapy are a therapeutic need.

Valproic acid (VPA) is a neurotherapeutic drug prescribed worldwide as therapy for seizures, bipolar disorder, and migraine, including children, adult and women of reproductive age. It is one of the major antiepileptic drugs in clinical practice and the drug of choice par excellence for all varieties of generalised epilepsy syndromes, primary or symptomatic [20]. Recently, VPA exerts protective effects for various neurological diseases, including spinal cord injury [21,22], stroke [23], traumatic brain injury [24], motor neuron diseases [25], Parkinson's disease [26], Alzheimer's disease [27] and Huntington's disease [28]. There is now accumulating evidence that VPA may have potential in the treatment of central nervous system disorders and the neuroprotective functions are linking with its inhibition on histone deacetylases (HDAC) [29,30]. It has recently been demonstrated that VPA robustly promotes neurite outgrowth, activates the extracellular signal regulated kinase pathway [30, 31,32]. However, the effect of VPA on the oxaliplatin-induced neuropathy remains unexplored.

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Accordingly, in the present study, we investigated the effect of VPA in prevention of oxaliplatin induced periphery neuropathy in the rat model. The anti-neuropathic role of VPA was evaluated in oxaliplatin-treated animals by analyzing pain behavior in relation to morphological and functional protection of the nervous system.

3. MATERIALS AND METHODS

1) Animals

Six-week-old male Sprague-Dawley rats weighing 200–250 g (Japan SLC, Shizuoka, Japan) were employed in the present study. Animals were housed in groups of three to four per cage (size 26 × 41 cm), fed a standard laboratory diet and tap water ad libitum, and kept at 25 ± 2°C with a 12 h light/dark cycle, light at 8 a.m. All experiments were approved by the Experimental Animal Care and Use Committee of Tokyo Metropolitan University according to the National Institutes of Health guidelines (Permit Number: A28-16, A29-8), and we followed International Association for the Study of Pain (IASP) Committee for Research and Ethical Issues guidelines for animal research [33]. All efforts were made to minimize animal suffering and to reduce the number of animals used.

2) Oxaliplatin model and pharmacological treatments

Oxaliplatin (Elplat®) was obtained from Yakult Co., Ltd. (Tokyo, Japan). VPA was purchased from Nippon Zoki Pharmaceutical Co. (Osaka, Japan).

The rats (n=18) were divided into three groups (6 rats in each group) and treated as follows. *Oxaliplatin group*: oxaliplatin (4 mg/kg) was injected in intraperitoneal injection (i.p.) in volumes of 10 ml/kg twice weekly for 4 weeks (Days 1, 2, 8, 9, 15, 16, 22 and 23) (Fig. 1). Oxaliplatin was dissolved in a 5% glucose-water solution. The dose of oxaliplatin followed previous reports [6, 34]. *Oxaliplatin + VPA group*: administration both oxaliplatin (as above) and VPA (300 mg/kg) daily twice a day for 4 weeks in i.p. The dose of VPA followed previous reports [35, 36]. *Control group*: injection of vehicle (5% glucose solution) instead of oxaliplatin and VPA.

3) Assessment of general toxicity

The measurement of the body weights of rats was performed on Days 0, 1, 2, 3, 8, 9, 10, 15, 16, 17, 22, 23, 24 and 28 in every groups, including on the day of treatment and immediately prior to sacrifice.

Rats were examined daily for abnormal clinical signs such as piloerection, hindlimb weakness, gait disturbance or gastrointestinal disorders such as diarrhea.

4) von Frey test for mechanical allodynia

The mechanical allodynia was assessed by von Frey test. The von Frey test was performed before the first drug administration (on Day 0) and on Days 5, 15, 21 and 28 (Fig. 1). On Days 5, 15 and 21, the test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. von Frey filaments (Aesthesio®, Precise Tactile Sensory Evaluator 20 pieces Kit with Carrying case, USA) ranging 1-15 g bending force were applied to the midplantar skin of each hind paw with each application held for 6 s. Fifty percent paw withdrawal thresholds were determined by a modification of up-down method that described by Kawashiri et al. [6] and Ushio et al. [37]. First, each hind paw was touched with some filaments from 1 g up to the force that rat exhibited the withdrawal response, in ascending order.

Next, the paw was touched with some filaments from 15 g down to the force that rat did not exhibit the response, in descending order. These up and down steps were repeated three times. Fifty percent thresholds were determined by average of the weakest force in each up or down step.

5) Acetone test for cold hyperalgesia

The cold hyperalgesia was assessed by acetone test. The acetone test was performed before the first drug administration and on the first day of drug administration (on Day 0 and Day 1) and on Days 5, 7, 14, 21 and 28 (Fig. 1) according to the method described by Kawashiri et al. [6] and Ushio et al. [37]. On Days 1, 5, 7, 14 and 21, test was performed before drug administration. Rats were placed in a clear plastic box (20 × 17 × 13 cm) with a wire mesh floor and allowed to habituate for 30 min prior to testing. Fifty microlitre of acetone (Wako Pure Chemical Ltd., #016-00346, Osaka, Japan) was sprayed onto the plantar skin of each hind paw three times with a micro sprayer, and

rats were observed for 40 s from the start of the acetone spray. The number of elevation of each hind paw was recorded.

6) Assay of sciatic nerve axonal degeneration

On Day 28, the rats were deeply anesthetized with pentobarbital (50 mg/kg), and transcardially perfused with phosphate-buffered saline (PBS) (0.1M, pH 7.4), followed by 2.5% (w/v) glutaraldehyde in PBS. The sciatic nerves were rapidly dissected, and the samples were kept overnight in the same fixative at 4 °C. The fixed fibers were post-fixed with 1% osmium tetroxide solution for 3 hours, dehydrated in a graded alcohol series, and embedded in EPON 815 (Wako, Japan). For light microscopy, semi-thin sections were cut from each block and stained with toluidine blue. The stained sections were observed using a light microscope (BX63, Olympus Corp., Tokyo, Japan). The density of axon area was calculated by image analysis software (ImageJ 1.50a; Wayne Rasband, National Institutes of Health, MD, USA).

7) Histopathological assessment on dorsal root ganglia

On Day 28, the dorsal root ganglion (DRGs) and spinal cord specimens (at segments L4 and L5) were excised from rats of each group, and fixed by immersion in 4% PFA overnight at 4°C. The tissues were then washed with PBS, dehydrated with ascending grades of reagent alcohol, cleared in two changes of xylene, infiltrated with paraffin, and sliced to 5 μm, mounted on charged slides. The DRG specimens were stained with Azan-Mallory method as manual, and the spinal cord specimens were performed with immunohistochemistry for GFAP and Iba1 as below 2.8.

Cellular dimensions of L4-L5 DRGs were measured using a method adapted from Di Cesare Mannelli et al. [3,38]. In these sections, using a 40x objective lens, the numbers of neurons with nuclei, nucleoli, multiple nucleoli, and nucleolar eccentricity (eccentric nucleolus) were counted. The nucleolus were considered eccentric when its center (or that of the largest one if there appeared to be more than one) lay in the outer half of the radius of the nucleus. The results were expressed as percentage of those cells with a visible nucleolus. Four consecutive

sections for each animal were analyzed. The reported data were obtained by averaging the data of L4 and L5 ganglia.

8) Immunohistochemical evaluation of GFAP and Iba1 in L4-L5 spinal cord

The immunohistochemical procedures were performed according to our previous study [39]. Briefly, after rinsing the fixed tissue specimens in 0.01 M PBS (pH 7.4), endogenous peroxidase activity was inhibited by 30-min incubation in methanol containing 0.3% (v/v) hydrogen peroxide. After rinsing in PBS, the sections were blocked with normal goat serum for 1 h at room temperature, were then incubated for overnight at 4°C in PBS containing the primary antibodies, against glial fibrillary acidic protein (GFAP; mouse, 1:300; MAB3402, Chemicon, Temecula, USA) for astrocyte staining and Iba1 (rabbit, 1:200; #019-19741, Wako Pure Chemicals, Osaka, Japan) for microglial staining. After rinsing in PBS, sections were incubated in donkey anti-mouse IgG secondary antibody labeled with Alexa Fluor 488 (1:2000, Thermo Fisher Scientific, Rockford, USA) and chicken anti-rabbit IgG secondary

antibody labeled with Alexa Fluor 488 (1:500, Thermo Fisher Scientific, Rockford, USA), respectively, at room temperature for 1 h.

Negative control sections (no exposure to the primary antisera) were processed concurrently with the other sections for all immunohistochemical studies. We obtained a single optical density value for the dorsal horns by averaging the two sides in each rat, and these values were compared to the homologous average values from the vehicle-treated animals.

9) Quantitative analyses of GFAP and Iba1 immunohistochemistry

Images were acquired by a motorized ZEISS ImagerM1 microscope equipped with a DS-Fi3 camera (Nikon, Tokyo, Japan). Morphological examination of astrocyte and microglia morphology was assessed by inspection of at least three fields (20 x) in the dorsal horn per section.

Quantitative analysis of GFAP and Iba1-positive cells was performed by collecting at least three independent fields through a 20 X 0.5NA objective. The densities of GFAP and Iba1-positive cells were calculated

by means of the automatic thresholding and segmentation features of ImageJ (ImageJ 1.50a; Wayne Rasband, National Institutes of Health, MD, USA). Results, given as the area fraction (%) occupied by the thresholded GFAP- or Iba1-positive cell number, respectively. Five spinal cord sections were analyzed for each animal.

10) Statistical analyses

Data are expressed as the mean ± standard devtion (SD). ANOVA and the Tukey's multiple comparison tests were employed for statistical analysis. All tests were performed as two-sided test and a p value of <0.05 was accepted as significant.

4. RESULTS

1) General toxicity of oxaliplatin

Rats were injected with either oxaliplatin (4 mg/kg) or VPA (300 mg/kg) at dosages corresponding to human chemotherapy, while 5% glucose solution was used as a control treatment (Fig. 1). No deterioration in general status was observed in any of the groups, and no rats died in the course of our experiments. No significant differences in body weight were observed between groups at any time (data not shown).

2) Effects of VPA on mechanical allodynia in oxaliplatin-induced neuropathy

Before the first oxaliplatin injection, there were no significant differences in withdrawal thresholds in all groups in the von Frey test. Oxaliplatin significantly reduced the withdrawal threshold compared with vehicle on Days 15, 21 and 28 ($P < 0.01$). Repeated administration of VPA significantly inhibited the oxaliplatin-induced

reduction of the withdrawal threshold on Days 21 and 28 ($P < 0.05$) (Fig. 2).

3) Effects of VPA on cold hyperalgesia in oxaliplatin-induced neuropathy

In the acetone test, there were no significant differences in number of withdrawal responses in all groups before the first oxaliplatin injection. Oxaliplatin significantly increased the number of withdrawal responses compared with vehicle on Days 1, 5, 7 and 14 ($P < 0.05$ or 0.01). However, no significant difference in withdrawal responses compared to the vehicle group was observed on Days 21 and 28 ($P > 0.05$). Repeated administration of VPA did not significantly inhibited the oxaliplatin-induced increase of the number of withdrawal responses on any days ($P > 0.05$) (Fig. 3).

4) Effect of VPA on oxaliplatin-induced histological change in rat sciatic nerve

Histological abnormalities in sciatic nerve were observed in vehicle-, oxaliplatin-treated and oxaliplatin + VPA-treated rats on Day 28. The

quantification analysis showed that oxaliplatin caused the decrease in the density of myelinated fibers and the degeneration of myelinated fibers in rat sciatic nerve ($P < 0.05$, Fig. 4B), and co-treatment with VPA had no effect on the oxaliplatin-induced decrease of the density of myelinated fibers ($P > 0.05$, Fig. 4C).

5) Effect of VPA on morphological derangement of DRG neurons

Morphologic and morphometric determinations on morphological derangement of DRG neurons were performed under the light microscope after Azan–Mallory stain. On day 28, oxaliplatin-induced damage was evidenced by the occurrence of multinucleolated neurons (Fig. 5B) and nucleolar eccentricity (Fig. 5B and C) distributed on small, medium and large neurons. VPA exerted a significant protective effect by reducing the occurrence of eccentric nucleolus neurons caused by oxaliplatin (Fig. 5D).

6) Effect of VPA treatment on glial cell activation profile in the spinal cord

To establish a relationship between pain relief and glial modulation the cell densities of astrocytes and microglia were calculated in the dorsal horn of the spinal cord using immunohistochemistry with antibodies against GFAP and Iba1, respectively.

In the spinal cord, repeated oxaliplatin injections (Day 28) induced an increase in GFAP-positive cells (Fig. 6B), astrocyte density increased over the entire surface of the spinal cord, particularly in the superficial laminae. VPA treatment prevented the increase in the density of the dorsal horn GFAP-positive cells (Fig. 6C).

The same as GFAP and shown in Fig. 7B, the number of Iba1-expressing cells in dorsal horn superficial laminae of oxaliplatin-treated rats was significantly increase than the vehicle group. VPA treatment prevented the increase in the density of the dorsal horn microglial cells (Fig. 7C).

5. DISCUSSION

In the present study, to demonstrate that VPA-treatment relieved the oxaliplatin-induced peripheral neuropathy, according to previous studies [10,12], oxaliplatin (4 mg/kg) was injected intraperitoneally to rats twice a week, and VPA (300 mg/kg) was administered daily twice a day for 4 weeks. The results were analysed using behavioral tests, and histopathological or immunohistochemical evaluations. In the von Frey test, VPA administration significantly attenuated the mechanical hyperalgesia induced by oxaliplatin injection. On the other hand, it was almost ineffective against the oxaliplatin-induced cold hyperalgesia in the acetone test. Therefore, it suggested that VPA has a protective effect on oxaliplatin-induced chronic peripheral neuropathy on the mechanical hyperalgesia.

VPA as a broad-spectrum HDAC inhibitor, is an anticonvulsant and mood-stabilizing drug with neuroprotective effects [40]. The HDAC inhibitors, such as trichostatin A and valproic acid, restored peripheral and systemic morphine analgesia in neuropathic pain. It suggests that

HDAC inhibitors could serve as adjuvant analgesics to morphine for the management of neuropathic pain [35]. A clinical investigation demonstrated sodium valproate is well-tolerated, and provides significant subjective improvement in painful diabetic neuropathy [41]. Oxaliplatin-induced peripheral neurotoxicity in the peripheral nerve shows several histological characteristics including demyelination and degeneration of nerve fibers, and decrease in the number of myelinated fibers [42]. In this study, oxaliplatin caused sciatic nerves of the oxaliplatin-treated group showed axonal degeneration and decreased density of myelinated fibers. However, these histological changes were not ameliorated in the tissue of rats treated with co-administration of oxaliplatin and VPA. It suggested that VPA was unable to prevent demyelination and degeneration of nerve fibers from oxaliplatin-induced peripheral neurotoxicity.

Oxaliplatin causes damage to cell bodies and selective atrophy of subpopulations of DRG neurons [43]. In previous evidence [44], it is demonstrated that DRGs are a primary target for oxaliplatin neurotoxicity. In the present study, the histological determinations

were performed on lumbar DRGs from oxaliplatin-treated rats, VPA exerted a significant protective effect by reducing the occurrence of multinucleolated neurons and the nucleolar eccentricity caused by oxaliplatin. VPA prevented morphological derangements in DRGs from oxaliplatin-treated rats, showed the same effects of N-Palmitoylethanolamine in oxaliplatin-treated rats[3].

Besides the neuronal damage, glial cells have recently been recognized as a powerful modulator of pain. The activation of spinal astrocytes has been reported to be involved in the oxaliplatin-induced neuropathic pain [45,46]. In models of trauma-induced neuropathy, microglia appear to exert a key role in the initial phases of neuropathic pain whereas astrocytes may be involved in its maintenance [47,48]. In addition, glial inhibitors have been described as pain relievers and glial cells are emerging as a new target for drug development [49]. The increased cell density of astrocyte and microglia is strongly related to pain hypersensitivity since the glial inhibitor minocycline and fluorocitrate fully prevent oxaliplatin-evoked pain [38].

N-Palmitoylethanolamine has been reported to modulate glial cells and exert antinociceptive effects on oxaliplatin-induced neuropathic pain in rats [3]. Furthermore, Kimura et al. [50,51] reported VPA (300mg/kg) was administered via intraperitoneal injection in a conditional knockout mice, which exhibit glaucomatous pathology including glutamate neurotoxicity and oxidative stress in the retina, daily for 2 weeks. It showed VPA prevents retinal degeneration in a murine model of normal tension glaucoma. Subcutaneous injections of 300 mg/kg VPA twice a day, VPA-mediated neuroprotection against I/R injury in the retina may involve cytoprotective Hsp70 induction via transcriptional activation and inhibition of the mitochondria-mediated apoptosis pathway [40]. In developing brain, few studies have examined VPA effects on glial cells, particularly astrocytes [36].

In the present study, to examine the effect of VPA on oxaliplatin-induced glial activation on spinal cord, immunohistochemical evaluation using GFAP and Iba1 antibodies was performed. On day 28, a lower pain threshold was accompanied by effects on spinal astrocytes and microglia that involve a significant increase of the number of cells

immunoreactive to GFAP and Iba1, respectively. The present results reveal an inhibitory effect of VPA on astrocytes and microglia in the dorsal horn of the spinal cord with decreasing in the number or activation of both cell types. It suggested that VPA-ameliorated oxaliplatin-induced astrocytes and microglia activation, meaning that proinflammatory mediator-related nociceptor sensitization could be prevented by VPA administration.

In conclusion, the present results demonstrate for the first time that VPA administration ameliorated the oxaliplatin-induced behavioral, biochemical and histopathological changes in rats. The VPA-mediated effects in this study may be attributed to neuroprotection properties and ameliorating oxaliplatin-induced astrocytes and microglial activation. VPA may offer a dual protective approach against etiological factors and resulting maladaptative plasticity. However, further study is needed to evaluate the effect of VPA on several symptoms of oxaliplatin-induced peripheral neuropathy and the mechanism of VPA on biomolecular changes of oxaliplatin-induced neuropathy. To get more conclusive results, a larger number of rats in each group and

more elaborate techniques are needed.

6. REFERENCES

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7. FIGURES AND LEGENDS

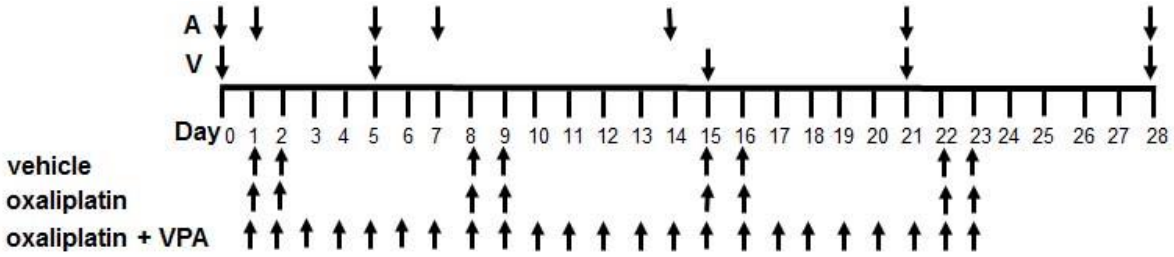

Figure 1. Schedual of drug administration and behavioral test.

A: Acetone test; V: von Frey test; vehicle: intraperitoneal injection (i.p.) with 5% glucose solution; oxaliplatin: oxaliplatin 4mg/kg i.p.; oxaliplatin + VPA group: oxaliplatin 4mg/kg and VPA 300mg/kg i.p., respectively.

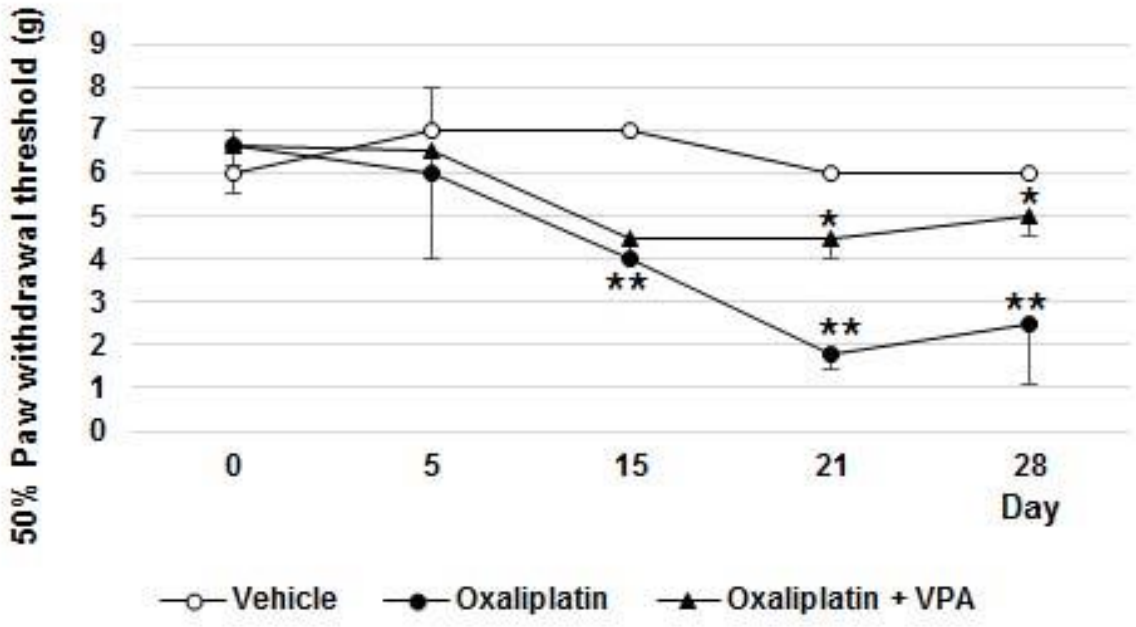

Figure 2. Effects of repeated administration of VPA on oxaliplatin-induced mechanical allodynia in von Frey test in rats.

Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks. VPA (300 mg/kg) was administered daily twice a day for 4 weeks. The von Frey test was performed before the first drug administration (on day 0) and on days 5, 15, 21 and 28. Values were expressed as the mean ± standard error mean of 6 animals. ∗∗P < 0.01 compared with vehicle. ∗P < 0.05 compared with oxaliplatin alone.

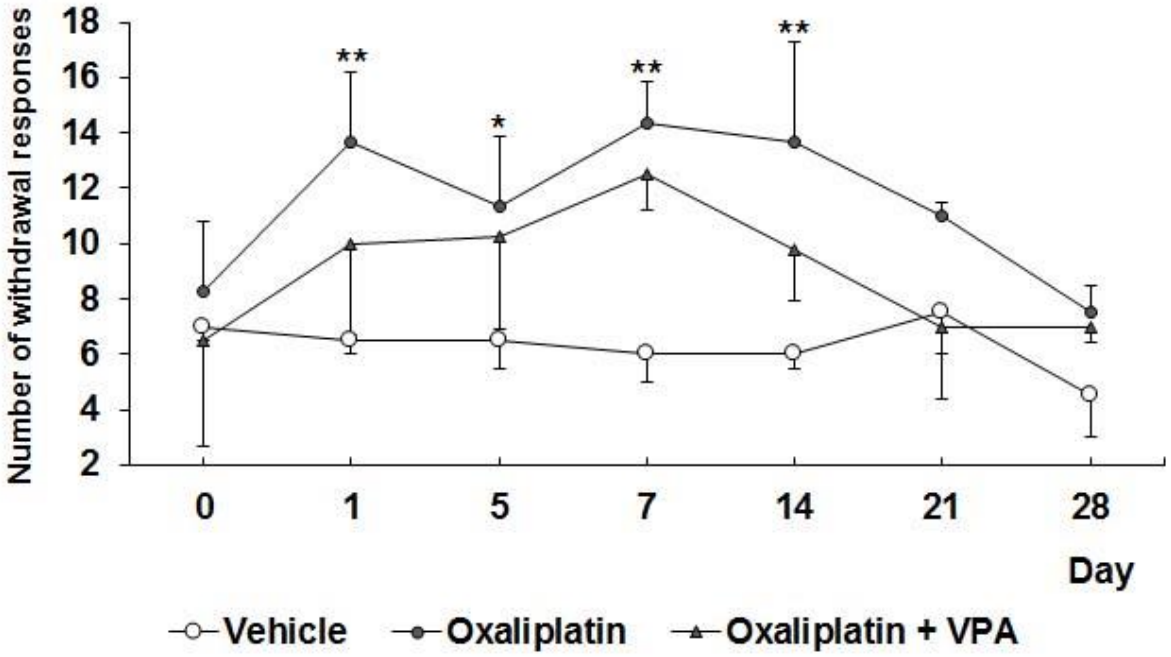

Figure 3. Effects of repeated administration of VPA on oxaliplatin-induced cold hyperalgesia in acetone test in rats.

Oxaliplatin (4 mg/kg) was administered i.p. twice a week for 4 weeks. VPA (300 mg/kg) was administered daily twice a day for 4 weeks. The acetone test was performed before the first drug administration (on day 0) and on days 1, 3, 5, 7, 14, 21 and 28. Values are expressed as the mean ± standard error mean of 6 animals. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with vehicle.

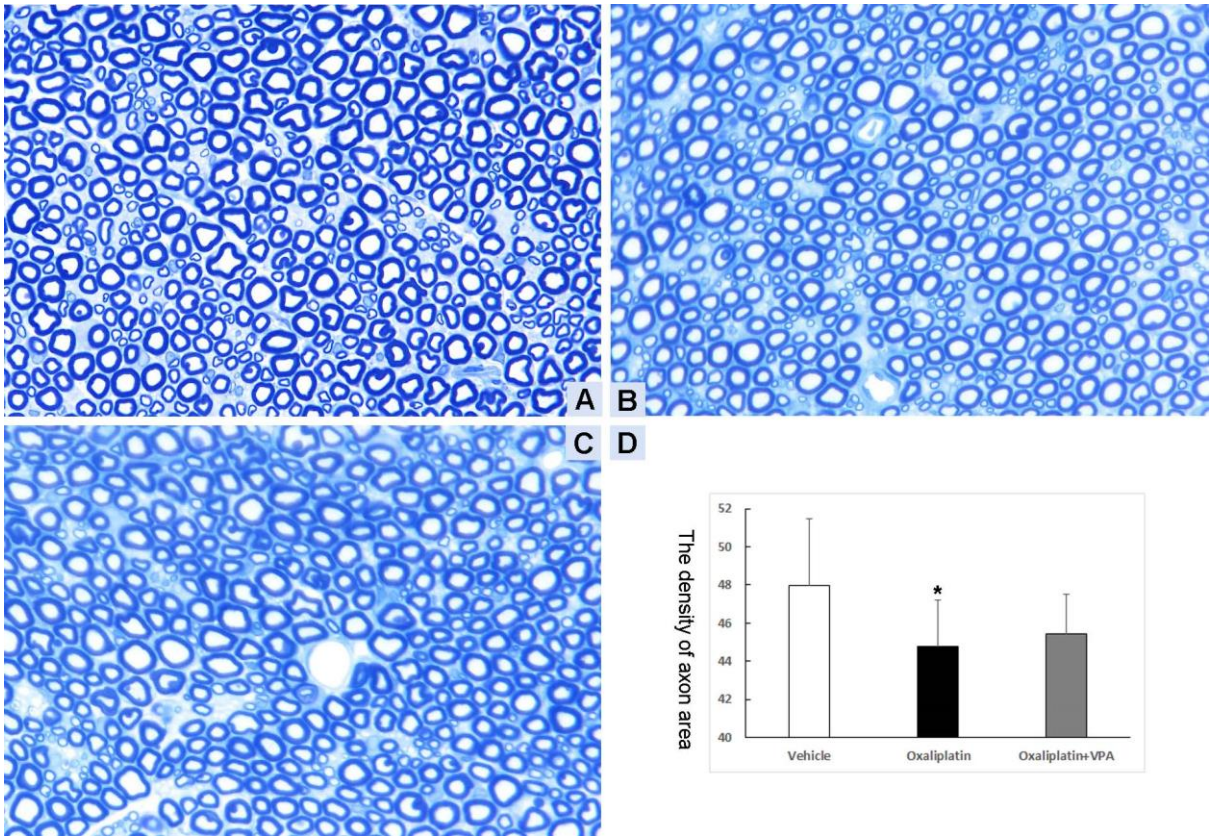

Figure 4. Effect of repeated administration of VPA on histological change induced by oxaliplatin (B) in rat sciatic nerve.

On day 28, The sciatic nerve was harvested, and samples were stained with toluidine blue. Images were captured at 800× magnification. The area of axon was calculated by image analysis software (ImageJ 1.50a). Values were expressed as the mean ± standard error mean of six animals. *$P < 0.05$ compared with vehicle (A). (C), oxiliplatin + VPA.

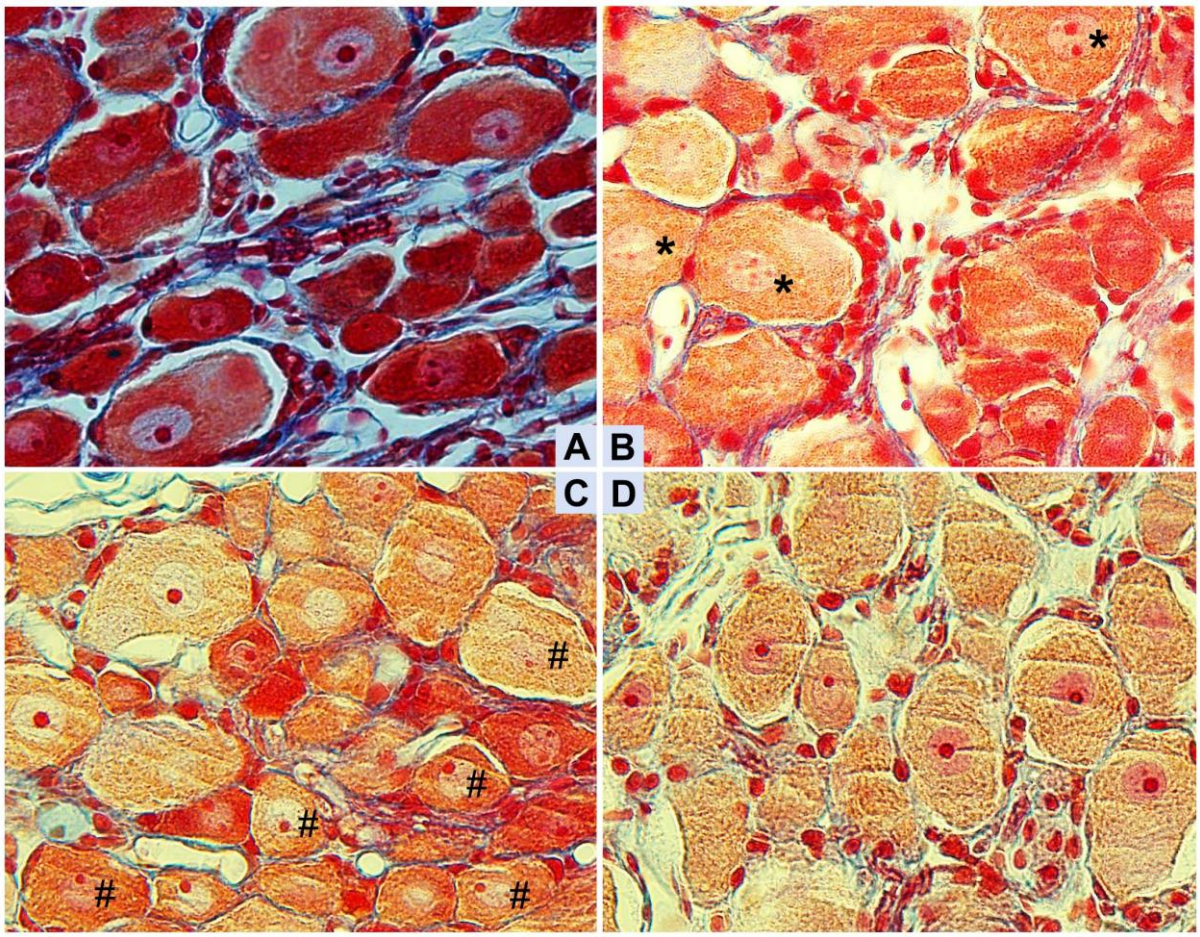

Figure 5. Morphological aspects of the peripheral nervous system. The protective effect of repeated administrations of VPA was evaluated on oxaliplatin-damaged DRGs on day 28. DRG sections were stained by the Azan-Mallory method. Light micrographs (original magnification 200 x) were analyzed by counting the incidence of eccentric nucleoli (#) and multinucleolated neurons (*).

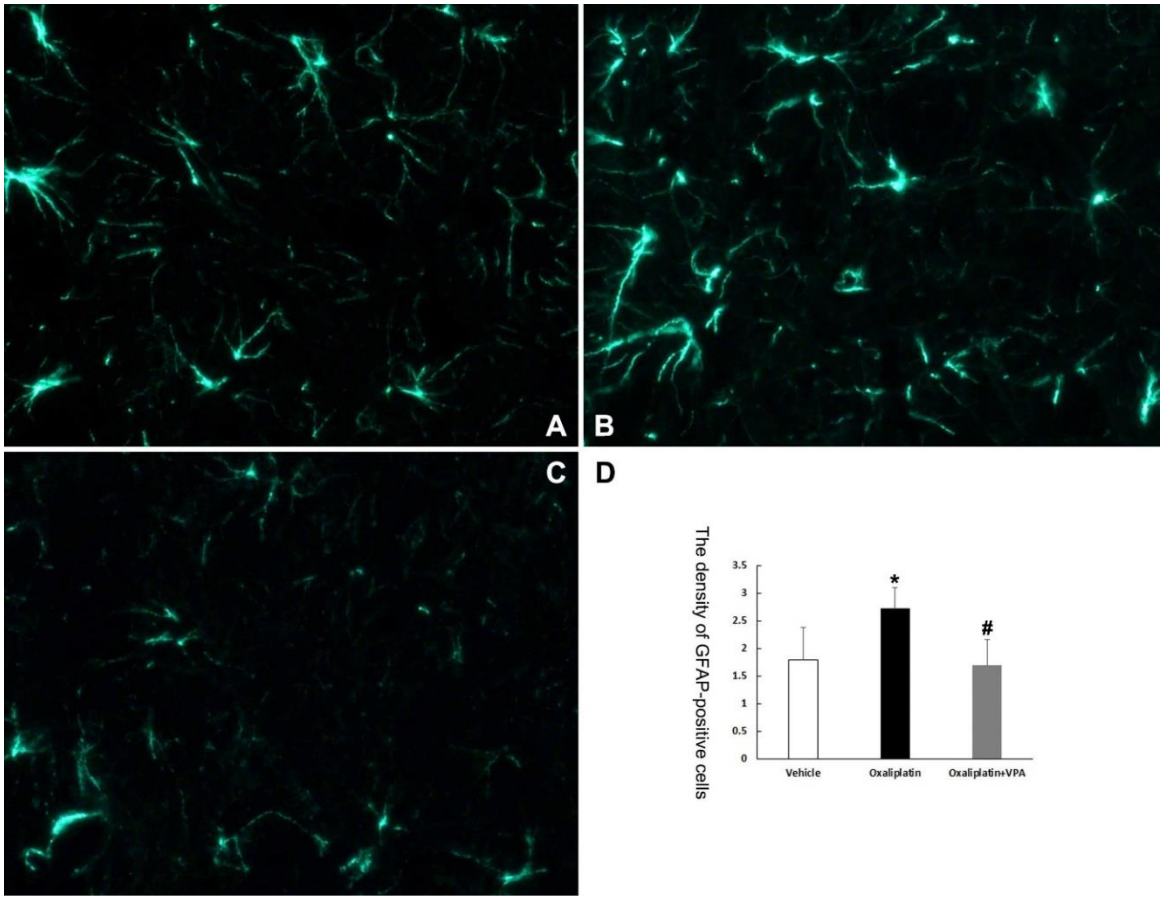

Figure 6. Astrocytes activation profile in the lumbar 4-5 spinal cord.

The effect of repeated treatment with VPA was evaluated in oxaliplatin-treated rats on day 28. The density of GFAP-positive cells was calculated by means of the automatic thresholding and segmentation features of ImageJ (ImageJ 1.50a; Wayne Rasband, National Institutes of Health, MD, USA) in (D). Images (original magnification 200 x) of sections showed vehicle in (A), oxaliplatin in (B) and oxaliplatin + VPA in (C). Each value represents the mean of 6

rats per group, performed in two different experimental sets.

*P<0.05 versus vehicle; $^{\#}$P<0.05 versus oxaliplatin.

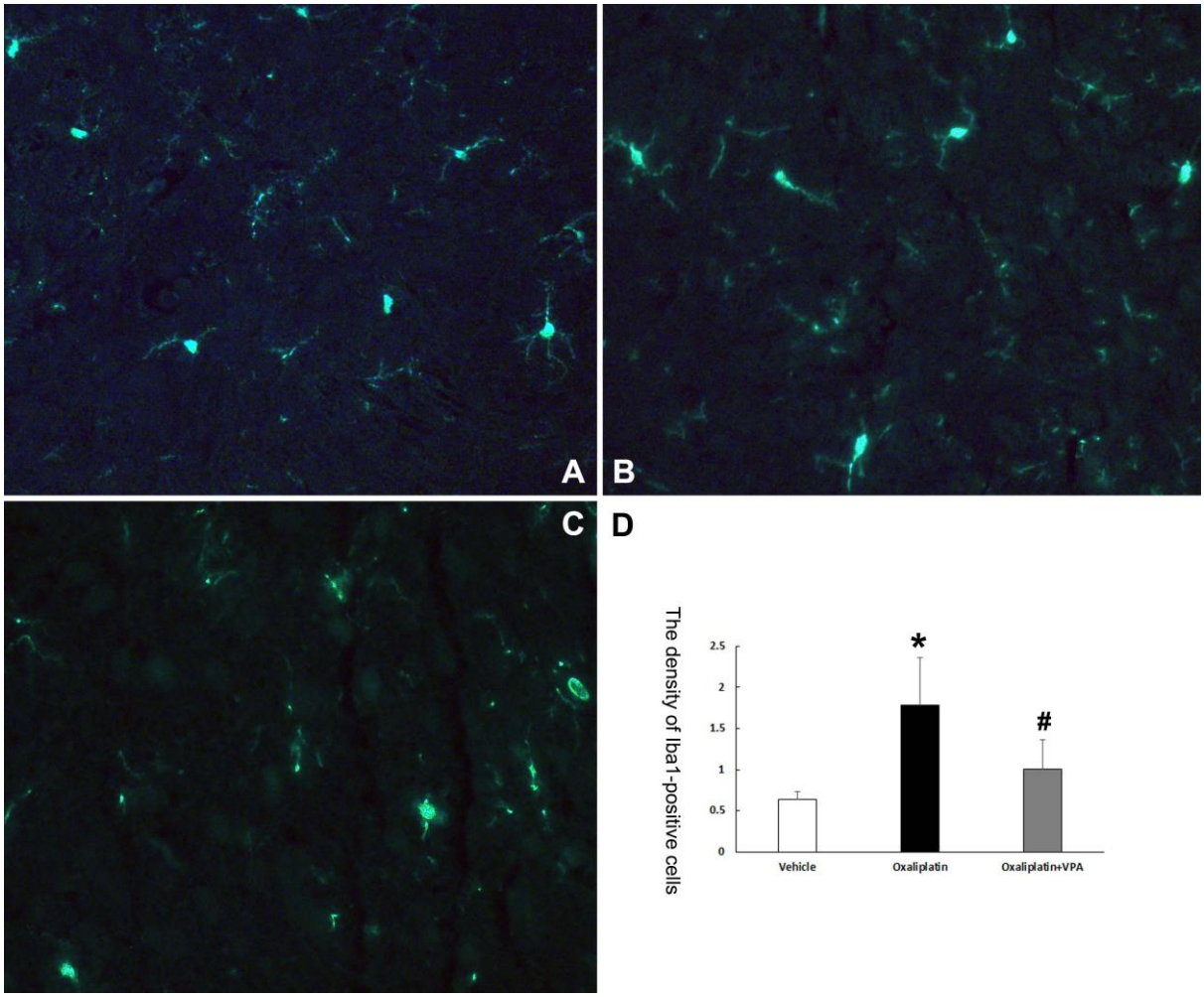

Figure 7. Microglia activation profile in the lumbar 4-5 spinal cord.

The effect of repeated treatment with VPA was evaluated in oxaliplatin-treated rats on day 28. The density of Iba1-positive cells was calculated by means of the automatic thresholding and segmentation features of ImageJ (ImageJ 1.50a; Wayne Rasband, National Institutes of Health, MD, USA) in (D). Images (original magnification 200 x) of sections showed vehicle in (A), oxaliplatin in

(B) and oxaliplatin + VPA in (C). Each value represents the mean of 6 rats per group, performed in two different experimental sets.

*P<0.05 versus vehicle; $^{\#}$P<0.05 versus oxaliplatin.

8. ACKNOWLEDGMENT

The authors wish to acknowledge Support Center for Medical Research and Education, Tokai University, for technical support of Department of Cell Biology and Histology, analysis with Nahoko Fukunishi and Masayoshi Tokunaga. Part of this study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Nos. 22590171 and 25460265), and a Grant for Scientific Research from Faculty of Health Sciences, Tokyo Metropolitan University (No.280530-1425000).

9. LIST OF PUBLICATIONS

Original Articles

1. Using a Whole-mount immunohistochemical method to study the innervation of the biliary tract in *Suncus murinus*
   Ren K, Dai Y, Yi K, Kinoshita M, Itoh M, Sakata I, Skai T, Yi SQ. J Vis Exp. 2017 Jun 15;(124). doi: 10.3791/55483.

2. Inhibitory effects of valproic acid in oxaliplatin-Induced neuropathy in rat model
   Ren K, Yi K, Dai Y, Fujiwara M, Ohta T, Terayama H, Sakabe K, Yi SQ. *J Gastroenterol Hepatol Res* 2017; 6(6): 2461-2469.

3. A new model for localized pancreatitis, ventral pancreatic duct ligation in the adult house musk shrew, *Suncus murinus*
   Ren K, Kinoshita M, Dai Y, Wang H, Yi K, Itoh M, Yi SQ. Academia J Biotechnol. 2016; 4(2): 055-061. doi: 10.15413/ajb.2015.0242

4. Innervation of extrahepatic biliary tract, with special reference to the direct bidirectional neural connections of the gallbladder, sphincter of Oddi and duodenum in *Suncus murinus*, in whole-mount immunohistochemical study
   Yi SQ, **Ren K**, Kinoshita M, Takano N, Itoh M, Ozaki N. Anat Histol Embryol. 2016 45(3):184-8. doi: 10.1111/ahe.12186.

5. 膵頭神経叢の分布形態の再検討

   易　勤、佐藤 厳、三輪 容子、**Ren K**、Dai YD、木下 正信、永川 裕一、土田 明彦.胆膵の病態生理 32, 2016.

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1. 膵臓の形態形成とその血管系
   易　勤，佐藤巌，三輪容子，**Ke Ren**，Yidan Dai，Kai Yi，藤原基，木下正信．第 122 回日本解剖学会総会・全国学術集会　2017 年 3 月 28～30 日　長崎大学坂本キャンパス（長崎）

2. Mesopancreas の本体は？
   易　勤，島田和幸，佐藤巌，三輪容子，**Ke Ren**，Yidan Dai，Kai Yi，藤原基，木下正信．第 122 回日本解剖学会総会・全国学術集会　2017 年 3 月 28～30 日　長崎大学坂本キャンパス（長崎）

3. 胎児ラットにおける上腕二頭筋腱と付着部の形態学的変化について
   藤原　基，伊藤　正明，Kai Yi，**Ke Ren**，Yidan Dai，木下　正信，易　勤。第 122 回日本解剖学会総会・全国学術集会　2017 年 3 月 28～30 日　長崎大学坂本キャンパス（長崎）

4. The distribution and ramification of the coronary artery in fetus pigs
   Yidan Dai, Kazuyuki Shimada, Kai Yi, **Ke Ren**, Motoi Fujiwara, Masanobu Kinoshita, Shuang-Qin Yi. 第 122 回日本解剖学会総会・全国学術集会　2017 年 3 月 28～30 日　長崎大学坂本キャンパス（長崎）

5. ブタの胸鎖乳突筋
   Ke Ren，木田雅彦，Kai Yi，Yidan Dai，藤原基，木下正信，易　勤．第 122 回日本解剖学会総会・全国学術集会　2017 年 3 月 28～30 日　長崎大学坂本キャンパス（長崎）

6. Inhibitory effect and mechanism of low-dose paclitaxel in chronic pancreatitis and pancreatic fibrosis

Dai YD, **Ren K**, Yi K, Kinoshita M, Yi SQ. 第 121 回日本解剖学会総会･全国学術集会、2016 年 3 月 28 日(月)-3 月 30 日(水)、ビッグパレットふくしま

7. The effect of valproic acid in prevention of oxaliplatin-induced neuropathy in the rat model
Yi K, **Ren K**, Hori K, Ozaki N, Dai YD, Kinoshita M, Itoh M, Ohta T, Yi SQ. 第 121 回日本解剖学会総会･全国学術集会、2016 年 3 月 28 日(月)-3 月 30 日(水)、ビッグパレットふくしま

8. ブタ胎児標本を用い解剖学実習
Ren K、今田 康大、Dai YD、木下 正信、易 勤. 第 121 回日本解剖学会総会･全国学術集会、2016 年 3 月 28 日(月)-3 月 30 日(水)、ビッグパレットふくしま

9. PD 術式による膵臓の神経分布様式の再認識
易 勤、佐藤 厳、三輪 容子、木下 正信、**Ren K**、Dai YD、Yi K、伊藤 正裕、永川 裕一、土田 明彦. 第 121 回日本解剖学会総会･全国学術集会、2016 年 3 月 28 日(月)-3 月 30 日(水)、ビッグパレットふくしま

10. Reinvestigation of the suspensory muscle of the duodenum in cadaver dissection
Ren K, Miwa Y, Sato I, Yi K, Dai YD, Kinoshita M, Yi SQ. 第 121 回日本解剖学会総会･全国学術集会、2016 年 3 月 28 日(月)-3 月 30 日(水)、ビッグパレットふくしま

11. Re-evaluation of the development in the rat shoulder joint
Takano N, Itoh M, **Ren K**, Kinoshita M, Yi SQ.･ 第 120 回日本解剖学会総会・全国学術集会　2015 年 3 月 20～23 日　京都府立医科大学（神戸国際会議場・展示場）

12. Inhibitory effects of Saiko-keishi-to (TJ-10) on pancreatitis-reduced pain in a rat nodel of chronic pancreatitis
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13. スンクスの脂肪移植および脂肪細胞分化誘導実験研究
易勤、山本靖彦、中村恒夫、**ニン　カ**、渡辺賢、木下正信、山本博、尾崎紀之。第 119 回日本解剖学会総会・全国学術集会　2014 年 3 月 27～29 日　自治医科大学（栃木県）

14. Absence of adipocyte precursor cells in mesentery of *Suncus murinus*
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