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

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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 peripheral 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.

CONCLUSION: 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 astrocytic and microglial activation. VPA may offer a dual protective approach against etiological factors and resulting maladaptive plasticity.

Key words: Oxaliplatin; Peripheral neuropathy; Mechanical allodynia; Valproic acid; Astrocyte and Microglia; Nucleolar eccentricity

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management of the advanced stages of metastatic colorectal cancer[3]. 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[31].

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[8,9]. 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[11], carbamazepine[11], phosphatidylcholine[12], N-palmitoylphosphatidylcholine[11], exenatide[13], and goshajinkigan[14]. 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[15], exenatide[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[21-23], stroke[24], 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[29,30]. However, the effect of VPA on the oxaliplatin-induced neuropathy remains unexplored.

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

**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[31]. 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) (Figure 1). Oxaliplatin was dissolved in a 5% glucose-water solution. The dose of oxaliplatin followed previous reports[8,20]. 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[18,20]. 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 and 21 and 28 (Figure 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 plantar 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[20] and Ushio et al[27]. 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 (Figure 1) according to the method described by Kawashiri et al[20] and Ushio et al[27]. On Days 1, 5, 7, 14 and 21,
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Figure 1 Schedule 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 (4mg/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 (4mg/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.

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 transected percutaneously 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[39]. In these sections, using a 40x objective lens, the numbers of neurons with nuclei, nucleoli, multiple nucleoli, and nucleoloe 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, 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
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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.

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 deviation (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.

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 (Figure 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) (Figure 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

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), oxaliplatin + VPA.
compared to the vehicle group was observed on Days 21 and 28 ($P > 0.05$). Repeated administration of VPA did not significantly inhibit the oxaliplatin-induced increase of the number of withdrawal responses on any days ($P > 0.05$) (Figure 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 ($P < 0.05$, Figure 4B), and co-treatment with VPA had no effect on the oxaliplatin-induced decrease of the density of myelinated fibers ($P > 0.05$, Figure 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 (Figure 5B) and nucleolar eccentricity (Figure 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 (Figure 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 (Figure 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 (Figure 6C).

The same as GFAP and shown in Figure 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 (Figure 7C).

DISCUSSION
In the present study, to demonstrate that VPA-treatment relieved the oxaliplatin-induced peripheral neuropathy, according to previous studies, oxaliplatin (4 mg/kg) was injected intraperitoneally to rats twice a week, and VPA (500 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. 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. A clinical investigation demonstrated sodium valproate is well-tolerated, and provides significant subjective improvement in painful diabetic neuropathy. 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. 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. In previous evidence, 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.

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. 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. In addition, glial inhibitors have been described as pain relievers and glial cells are emerging as a new target for drug development. The increased cell density of astrocyte and microglia is strongly related to pain hypersensitivity since the glial inhibitor minocycline and fluoroacetate fully prevent oxaliplatin-evoked pain.

N-Palmitoylethanolamine has been reported to modulate glial cells and exert antinociceptive effects on oxaliplatin-induced neuropathic pain in rats. Furthermore, Kimura et al. reported VPA (300 mg/}

**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.
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 microglial 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 maladaptive 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.

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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.
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