Vincristine Leads to Colonic Myenteric Neurons Injury via Stimulating M1 Macrophages Polarization

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Research

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Abstract

Background

Vincristine is widely used in treatment of various malignant tumors. The clinical application of vincristine is accompanied by peripheral neurotoxicity. The effect of vincristine on enteric neurons and the underlying mechanism are still unclear.

Methods

C57BL6/J mice were systematically treated with vincristine for 10 days, and macrophages were depleted using clodronate liposomes. The colonic myenteric plexus neurons were extracted. Macrophages from different parts were extracted in an improved way.

Results

In the current study, we demonstrated that system treatment of vincristine resulted in colonic myenteric neurons injury, proinflammatory factors increase and total gastrointestinal transport time increase. Vincristine promoted the M1-type macrophages polarization individually or in coordination with LPS and increased proinflammatory factors IL-1 β , IL-6, TNF- α via increasing the phosphorylation of ERK1/2 and p38. In addition, these proinflammatory factors led to colonic myenteric neurons apoptosis targeting on SGK1-FOXO3 pathway. Importantly, macrophage depletion alleviated colonic myenteric neurons injury and the increase of proinflammatory factors caused by system treatment of vincristine.

Conclusions

System treatment of vincristine led to colonic myenteric neurons injury via stimulating M1 macrophages polarization which was alleviated by depletion of macrophages.

Introduction

Vincristine is an alkaloid extracted from the plant vinca. Vincristine inhibits the division and proliferation of tumor cells by inhibiting the polymerization of micro-tubulin, leading to the death of tumor cells [1, 2]. Vincristine is widely used in treatment of various malignant tumors, including acute lymphocytic leukemia, lymphoma, etc. [3–5]. It is one of the anti-tumor drugs commonly used in clinic. However, the clinical application of vincristine is accompanied by serious side effects, among which peripheral neurotoxicity is particularly significant [3, 6, 7], which is characterized by sensory and motor dysfunction [8] and is obviously dose-dependent. This is the main reason for many patients to stop vincristine treatment and is one of the significant factors affecting the survival of cancer patients. As for the mechanism of vincristine peripheral neurotoxicity, it was initially believed that vincristine directly inhibited the polymerization of neuronal tubulin, interfered with the axoplasmic transport of neurons, and led to swelling of nerve fibers and axonal injury [9]. However, it has been recently suggested that most neurons are in a non-mitotic state, that the microtubules are not active. Therefore, the peripheral neurotoxicity

caused by vincristine might not be strictly related to the mechanism of anti-tumor action. There are several possible mechanisms including changing the plasticity of synaptic connections of neurons [10, 11]; activating glial cells [12–14]; reducing endogenous opioid peptide level [15]; changing Ca^{2+} homeostasis [16]; promoting inflammation [17–19]. Therefore, this requires us to further investigate the potential mechanism of the peripheral neurotoxicity caused by vincristine, so as to find innovative targets to alleviate peripheral neurotoxicity, which can help improve the tolerance of vincristine to cancer treatment, and improve the survival rate and quality of life of patients.

The enteric nervous system (ENS) is composed of the ganglion formed by the aggregation of neurons and nerve fibers. Neurons connect with each other forming nervous system which integrates and processes information independently, known as the "micro-brain" of the gastrointestinal tract [20], regulating important gastrointestinal functions, including movement, secretion, local immunity and inflammation. Anatomically, the intestinal tissue can be divided into mesentery, serous membrane, muscularis, submucosa, mucosa and epithelium [21]. The muscular layer is composed of the outer longitudinal muscle layer and the inner teres muscle layer. Between the two muscular layers is the longitudinal muscle myenteric plexus (LMMP) [21], which is responsible for the movement of the intestine; below the mucosa is the submucosal plexus, which is responsible for regulating intestinal sensations and secretion. It has been pointed out that the LMMP injury caused by vincristine may be related to the decrease of gastrointestinal peristalsis. After vincristine treatment, up to 57% patients have digestive symptoms such as constipation [22, 23].

There are also a large number of immune cells in the intestine, among which macrophages are particularly important. Macrophages are distributed in different layers of intestine, including the mucosa, submucosa and muscularis, in which the muscularis contain a large number of tissue-resident macrophages, called muscularis macrophages (MMs). MMs is located near the enteric ganglion, maintaining enteric homeostasis [24–26]. Macrophages can be polarized into different phenotypes under the effect of different cytokines in the micro-environment, and can be divided into classically activated (M1-type) macrophages and alternatively activated (M2-type) macrophages. Macrophages are polarized into M1-type macrophages when stimulated by IFN- γ and LPS, and secrete a large number of proinflammatory factors, such as IL-1 β , TNF- α , IL-6, which play an important part in host defense. On the contrary, macrophages are polarized into M2-type macrophages under the stimulation of IL-4 and IL-13 and produce anti-inflammatory mediators, such as IL-10, CD206 and Arg1, which can inhibit inflammation and promote wound healing [27]. Macrophages have strong plasticity and can be transformed between different phenotypes, and increase or decrease of a certain phenotype can lead to changes in homeostasis and the occurrence of disease. Some studies have revealed that the cross-talk between immune cells and intestinal neurons plays an important role between them [21]. A certain extent of communication between macrophages and intestinal neurons is essential for maintaining homeostasis and completing digestion and absorption in the digestive tract [24, 26, 28].

Vincristine, as a chemotherapeutic drug, is cytotoxic in nature, leading to the release of intracellular substances, which can stimulate the immune system and trigger the production of inflammatory

cytokines, which is also widely recognized [1, 29]. It has been reported that vincristine induced hyperalgesia reaction can be reduced by using clodronate liposomes to deplete macrophages [18]. In vincristine induced painful neuropathy, dorsal root ganglion and sciatic nerve macrophage elevations and mechanical allergies occur simultaneously [18, 30], accompanied by increases in IL-1 β , IL-6, TNF- α , CX3CR1, CCL2 [30–32]. However, the effect of vincristine on enteric structure and movement and its mechanism have not been studied in depth, especially the changes of neurons as well as the interaction between neurons and immune cells after treatment of vincristine. Therefore, in this paper, we mainly focused on the effect of vincristine on enteric macrophages, as well as its role and mechanism in enteric neurons injury, so as to develop treatment or prevention strategies to improve the survival rate and quality of cancer patients. Through the study, we found that the injury of colonic myenteric neurons induced by vincristine was generated by regulating the M1-type macrophages polarization and increasing proinflammatory factors such as IL-6, IL-1 β and TNF- α . These proinflammatory factors led to the apoptosis of colonic myenteric neurons by SGK1-FOXO3 pathway. Therefore, controlling the direction of macrophage polarization and the release of proinflammatory factors may be a new target for enteric myenteric neurons injury in system treatment of vincristine.

Materials And Methods

Reagents

Vincristine sulfate and Lipopolysaccharide (LPS) were purchased from Sigma-Aldrich (St. Louis, MO). IL-4 was obtained from R&D Systems (Minneapolis, MN). Clodronate liposomes and Control liposomes were purchased from FormuMax (Silicon Valley, CA). Antibodies for β -Tubulin, p44/42, phospho-p44/42, p38, phospho-p38, SGK1, phospho-SGK1, FOXO3, phospho-FOXO3, cleaved casepase-3 and GAPDH were acquired from Cell Signaling Technology (Danvers, MA) and Abcam (Cambridge, U.K.). The secondary antibodies were obtained from Abcam (Cambridge, U.K.), Invitrogen Life Technology (Foster City, CA) and Zhongshan Golden Bridge Biotechnology (Beijing, China). Anti-mouse CD86-PerCP/Cy5.5 or CD206-PE antibody were purchased from Biolegend (San Diego, CA). Mouse ELISA kits were got from Dakewe Biotech (Shenzhen, China).

Experimental animals

Male C57BL6/J mice, 6-8 weeks old, were obtained from the Animal Center of Shandong University. All experimental mice were taken care of in a pathogen-free environment and housed in groups randomly in standard cages (5–6 mice per cage) and drunk water and ingested food optionally. The experimental mice rooms were kept the environment temperature at 22-24°C under 12-hour light/dark cycle. All mice experiments were conducted in the Animal Center of Shandong University, with the approve of the Medical Ethics Committee for Experimental Animals (ECSBMSSDU2020-2-006). The suffering and number of mice was maintained to the minimum.

Drug administration and treatment

Vincristine sulfate (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile saline, and was intraperitoneal injection (i.p.) to mice in a dose of 0.1 mg/kg once per day for 10 consecutive days, which has been generally proved to be a feasible system treatment of vincristine [33-36]. All experiments were carried out one day after the last injection of vincristine.

Clodronate liposomes (200 μ L of the order preparation; FormuMax, Silicon Valley, CA) were used to deplete macrophages in mice and had been shown could deplete about 90% macrophages within 24 to 36 hours after systemic administration [37-39]. Control liposomes (200 μ L of the order preparation; FormuMax) and saline were used as a control. These two liposomes were intraperitoneally injected the day before the vincristine treatment was started, and then repeated every 3 days [40, 41]. This strategy ensured persistent depletion of macrophages throughout the vincristine treatment period.

The first experiment was divided into two groups (N=6/group): control group and vincristine treatment group. The second experiment was divided into four groups (N=6/group): (1) Control Liposomes, (2) Control Liposomes + VCR (non-depleted macrophages vincristine treatment group), (3) Clodronate Liposomes (only depleted macrophages group), (4) Clodronate Liposomes + VCR (depleted macrophages vincristine treatment group). The experimental schedule and cell/tissue analyses for the first experiment is shown in Supplemental Figure 1A. The experimental schedule for the second experiment is shown in Supplemental Figure 1B.

Colonic myenteric neurons isolation and culture

The acute isolation and culture of colonic myenteric neurons were carried out in accordance with the methods explored in our laboratory [42]. C57BL6/J mice were euthanized with sodium pentobarbital (200mg/kg) and their colons were removed and quickly placed in dissecting dishes containing Krebs saline. The Krebs saline included following elements (in mM): KCl 5.9, NaH_2PO_4 1.2, MgCl_2 1.2, NaCl 120.6, NaHCO_3 15.4, D(+)-Glucose 11.5, CaCl_2 2.5. The mesentery was cut off with Venus scissors and the colonic segment was longitudinally slit along the mesenteric junction. The colon wall was spread out to fully expose the lumen and pinned well. The contents of the intestinal cavity were removed and rinsed thoroughly. The mucosa, submucosa, serosal layer, and circular muscle layers were carefully got rid under the microscope, leaving the LMMP for use. The LMMP was placed in Krebs saline containing with papain (10mg/ml, Solarbio, Beijing, China) at 37 °C for 55 minutes. The LMMP was cut into pieces with scissors, and then placed in the DMEM (Gibco, Foster City, CA) supplemented 10% heat-inactivated fetal bovine serum (FBS) (Gibco, Foster City, CA) and 1% penicillin–streptomycin solutions (Gibco) containing collagenase Σ (1mg/mL, Gibco) in asepsis pipes and digested for 55 minutes at 37°C. After digestion, the above medium was added in asepsis pipes to terminate and centrifuged at 1000 RPM for 7 minutes. The sediment was collected and suspended in the medium described above. Cells then were cultured in a incubator with 5% CO_2 at 37°C for 6 days. The fresh medium was changed daily.

RAW264.7 Macrophage-like cell lines culture

RAW264.7 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were seeded on a 24-well plate using the DMEM above. The cells (N=6/group) were treated with or without vincristine (10^{-9} M) for 30 minutes before IL-4 (10 ng/ml) or LPS (100 ng/ml) was added. The cells and supernatants were collected at a specified time for future experiments.

Peritoneal macrophages isolation and culture

The method of peritoneal macrophages isolation and culture was adopted from the method of previous studies [43, 44]. C57BL6/J mice were euthanized with sodium pentobarbital (200mg/kg) and skin surfaces were disinfected with 75% ethanol (N=6/group). The abdominal wall of the mouse was cut along the midline one layer with scissors. 5mL of precooled PBS containing 3% FBS was injected into the abdominal cavity of the mice with a 27g needle. The mice were then placed on ice block and massaged for 20 minutes. Peritoneal fluid was then carefully collected with a 25g needle and centrifuged at 1500 RPM for 7 minutes. The cells were collected in the precipitate. The precipitate was resuspend with DMEM above and cultured in a standard incubator with 5% CO₂ at 37°C. The cells were treated with or without vincristine (10^{-9} M) for 30 minutes before LPS (100 ng/ml) was added. After 2 hours, peritoneal macrophages were rinsed with sterile PBS for 3 times and replaced with fresh medium. After 12 hours, the supernatant was taken for the incubation of neurons for 48 hours (Figure 3A). The experiment was divided into four groups (N=6/group): (1) Control group (CM_{M0}), (2) single stimulation of vincristine group (10^{-9} M, CM_{M0+VCR}), (3) single stimulation of LPS group (100 ng/ml, CM_{M1}), (4) stimulation of vincristine combined with LPS group (CM_{M1+VCR}).

Colonic mononuclear cells isolation

Colonic mononuclear cell isolation was modified with the previous method [45]. C57BL6/J mice (N=7/group) were euthanized and their colons were quickly removed and placed in pre-cooled PBS. Colon contents were flushed with pre-cooled PBS. The colons were opened along the mesentery and cut into pieces. The pieces were placed in tubes containing 1mM DTT (Sigma-Aldrich), 1nM EDTA (Sigma-Aldrich), 1% penicillin–streptomycin, and 5% fetal calf serum in PBS and twice shaken in 37°C for 20 minutes. The supernatant was discarded. The tissue pieces were kept, washed with PBS for 3 times, cut into mince, and placed in RPMI 1640 with 1 mg/ml collagenase IV (Roche, Germany) to digest 50 minutes at 37°C. The tissue was terminated digesting by adding 5% fetal calf serum, then filtered by a 40 um cell filter, centrifuged at 1500 RPM for 10 minutes, retain the precipitation. Gradient centrifugation was performed on 40% and 75% Percoll (GE Healthcare, Sweden), at 1800 RPM for 20 minutes at 20°C. The middle layer was the mononuclear cell of the colon. Monocytes were collected and re-suspended with PBS or medium for the next experiment.

Immunofluorescence staining

Cells (N=4 or 6 /group) were fixed at room temperature with 4% paraformaldehyde for 25 to 30 minutes and washed with PBS and then sealed with 10% donkey serum at room temperature for 60 minutes. The

cells were incubated with mouse anti- β -Tubulin (1:1500, Abcam, Cambridge, U.K.) primary antibodies dissolved in 10% donkey serum overnight at 4°C. Similarly, tissue sections embedded in paraffin wax were placed in an oven for 2 hours to dewax. Then slices were boiled in sodium citrate buffer (10 mM, Beyotime, Shanghai, China) to repair for 25 minutes. The slices were cooled to room temperature and sealed with 10% donkey serum for 60 minutes. The tissue was incubated with rabbit anti- β -Tubulin (1:500, Abcam) primary antibodies dissolved in 10% donkey serum overnight at 4°C. Then slices were washed with PBS three times, 5 minutes each. The cells or tissue slides were incubated at room temperature with Alexa Fluor 568 (or 488) donkey anti-rabbit (1:2000, Invitrogen) secondary antibodies for 45 minutes. The nucleus were dyed with DAPI (1:1000, Beyotime) at room temperature for 5 minutes. Images of cells and tissues were observed and taken by fluorescence microscopy (Olympus IX71).

RNA extraction and quantitative real-time PCR

Total RNA from colonic tissues and cells (N=5 or 6 /group) was extracted according to the instructions using the cell/tissue rapid extraction kit (Aidlab Biotechnologies, Beijing, China). The total RNA was reverse-transcribed to cDNA with the Takara PCR Thermal Cycler SP (Takara Bio, Shiga, Japan). The expression of mRNA was presented as relative ratio to that of the housekeeping gene. The primers were composed by The Beijing Genomics Institute (Shenzhen, China) and used for qRT-PCR as showing in Supplemental Table1.

Flow cytometry

The proportion of proinflammatory and anti-inflammatory colonic mononuclear cells (N=7/group) was assessed by staining with anti-mouse CD86-PerCP/Cy5.5 or CD206-PE antibody (Biolegend, San Diego, CA). Flow cytometry analysis was carried out with flow cytometry C6 (BD Biosciences).

Western blotting analysis

Macrophages or colonic LMMP proteins (N=6/group) were collected in a RIPA-Lysis Buffer (Bioster Bio, Pleasanton, CA) containing 1% PMSF (Beyotime) and 2% PI (Beyotime). Equal amounts of protein were electrophoretic transferred to the PVDF membrane. 5% skimmed milk was dissolved in tween/tris-buffered salt solution. The membranes were blocked for 1 hour and then incubated with rabbit anti-p44/42 (1:1000, CST, Danvers, MA), anti-phospho-p44/42 (1:1000, CST), anti-p38 (1:1000, CST), anti-phospho-p38 (1:1000, CST), anti-SGK1 (1:1000, Abcam), anti-phospho-SGK1 (1:1000, Abcam), anti-FOXO3 (1:1000, Abcam) antibodies, anti-phospho-FOXO3 (1:1000, Abcam), anti-cleaved casepase-3 (1:1000, CST) and anti-GAPDH (1:2000, CST) antibody overnight at 4°C. After washed with tween/tris-buffered salt solution for 3 times, membranes were incubated with goat anti-rabbit IgG secondary antibodies (1:2000, Beyotime) conjugated with HRP at room temperature for 1 hours. Then the membranes were probed with an ECL Plus (Beyotime, China). Signal intensities of the protein immunoblot images were analyzed by ImageJ software.

Enzyme-linked immunoassay (ELISA)

Cell supernatant or tissue protein (N=4 or 6 /group) was collected after drug incubation or animal vincristine treatment. According to the specification, the specific protein concentration was detected by ELISA precoating kit (Dakewe Biotech, Shenzhen, China).

TUNEL staining

To quantify of neurons apoptosis, double staining of β -Tubulin (red) and TUNEL (green) were performed in Apoptosis Detection Kit (Servicebio, Wuhan, China) according to the specification (N=6/group). Datas were expressed with the proportion of TUNEL⁺ neurons (%) in every 10⁴ μ m² ganglion region.

Transcriptome RNA-seq analysis

After systematically treated with vincristine, the two groups (N=3/group) of LMMP with or without depleted macrophage were taken out and total RNA was extracted. RNA was tested for degradation and contamination. Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, MA) following the specification. Sequencing and data analysis were performed by Genechem (Shanghai, China). Differential expression genes of two groups were performed by DESeq2. The corrected *P*-value of 0.05 was used as the threshold of differential genes expression.

Gastrointestinal transit times (GITT)

A 0.5% methyl cellulose (Sigma-Aldrich) solution was prepared. After filtering with a filter, 6% (w/v) carmine red (Sigma-Aldrich) solution was dissolved in it. At the last day of vincristine treatment, the mice (N=6 or 7/group) were housed individually. After a night of fasting, mice were given 0.3 ml of carmine red solution and their excrement was observed every 5-15 minutes. Until the first red stool appears, the duration is the total gastrointestinal transport time.

Statistical analysis

All data were expressed as the mean and SEM. The group of the data was performed by analysis of ANOVA with Newman-Keuls or two-tailed Student's t-test. Statistical analysis was performed with GraphPad Prism version 5 (La Jolla, CA). *P*<0.05 was considered as statistically significant.

Results

Vincristine resulted in colonic myenteric neurons injury and proinflammatory factors increase

Vincristine or sterile saline was administrated intraperitoneally to mice in a dose of 0.1 mg/kg once per day for 10 consecutive days. The acute isolated colonic myenteric neurons were carried out and cultured for 6 days *in vitro*. Compared with control group, in vincristine treatment group, the number of neurons was reduced (69.28 ± 6.24 vs 103.53 ± 5.64 , *p*<0.01, n=6, Figure 1A, B) and the length of axons was shortened (41.32 ± 2.12 μ m vs 69.96 ± 3.35 μ m, *p*<0.001, n=6, Figure 1A, C). In the vincristine treatment group, total gastrointestinal transport time was longer than control group (233.67 ± 31.34 min vs 149.17

± 9.94 min, $p < 0.05$, $n = 6$, Figure 1D). Compared with control group, vincristine treatment group showed statistically significant decrease in CD206⁺ colonic mononuclear cells (24.11 ± 1.43 % vs 29.23 ± 1.39 %, $p < 0.05$, $n = 7$, Figure 1E, F) and increase in CD86⁺ colonic mononuclear cells (0.03 ± 0.17 % vs 0.44 ± 1.05 %, $p < 0.01$, $n = 7$, Figure 1G, H). We examined the expression of cytokines in the colonic LMMP, found that in vincristine treatment group, the mRNA levels of proinflammatory cytokines IL-1 β , IL-6 and TNF- α were increased to 1.74 ± 0.13 ($p < 0.01$), 1.78 ± 0.17 ($p < 0.01$), 1.81 ± 0.27 ($p < 0.05$) times ($n = 6$, Figure 1I), protein levels of proinflammatory cytokines IL-1 β , IL-6 and TNF- α were also increased (1583.43 ± 236.19 pg/ml vs 478.9 ± 29.62 pg/ml, 1593.89 ± 176.16 pg/ml vs 697.57 ± 51.48 pg/ml, 2320.76 ± 159.45 pg/ml vs 489.58 ± 76.88 pg/ml, $p < 0.001$, $n = 6$, Figure 1J). Anti-inflammatory factors Arg1 and IL-10 showed a downward trend ($p > 0.05$, $n = 6$, Supplemental Figure 2) compared with control group. This results suggested that the colonic myenteric neurons injury in the vincristine treatment group might be related to the increase of proinflammatory factors.

Vincristine promoted the M1-type polarization of RAW264.7 cells

It is reported that macrophages are densely packed around the myenteric neurons, which is significant for maintaining the normal physiological function of the intestine [24-26]. We first tested the dose-response of vincristine on the production of proinflammatory factors by RAW264.7 cells. Vincristine was used at different concentrations (10^{-10} M- 10^{-8} M), and 10^{-9} M vincristine was found to stimulate cells to produce proinflammatory factors IL-1 β (3.02 ± 0.54 times, $p < 0.01$, $n = 6$, Figure 2A) and IL-6 (3.92 ± 0.65 times, $p < 0.001$, $n = 6$, Figure 2B) with maximum efficacy. Next, we stimulated RAW264.7 cells with LPS (100 ng/ml) alone or in combination with vincristine at different concentrations (10^{-10} M- 10^{-8} M). The results showed that 10^{-9} M vincristine cooperated with LPS to promote M1-type macrophages polarization and produced proinflammatory factors IL-1 β (220.01 ± 8.24 times, $p < 0.001$, $n = 6$, Figure 2C) and IL-6 (89.68 ± 9.91 times, $p < 0.05$, $n = 6$, Figure 2D) with maximum efficacy. Subsequently, we stimulated RAW264.7 cells with vincristine (10^{-9} M) alone or in combination with LPS (100 ng/ml) and showed an increase in proinflammatory factors (IL-1 β , IL-6, TNF- α) expression in both mRNA and protein levels, but this increase was more pronounced when stimulated in combination with LPS compared with vincristine alone (Figure 2F-H). These findings indicated that the increase of proinflammatory factors in LMMP in mice systematically treated with vincristine maybe caused by the stimulation of colonic macrophages.

Supernatant of vincristine stimulated peritoneal macrophages induced colonic myenteric neurons injury *in vitro*

To further demonstrate whether the proinflammatory factors produced by vincristine stimulation of macrophages cause colonic myenteric neurons injury, we conducted *in vitro* experiments. The colonic myenteric neurons were incubated with peritoneal macrophages supernatant (conditioned medium, CM) with or without the pretreatment of vincristine and/or LPS. Compared with control group (CM_{M0}), the number of neurons were decreased by the supernatant of macrophages in vincristine (CM_{M0+VCR}, 11.78 ± 0.99 vs 27.65 ± 2.55 , $p < 0.001$, $n = 6$) and LPS (CM_{M1}, 10.60 ± 0.37 vs 27.65 ± 2.55 , $p < 0.001$, $n = 6$) and

axonal length of neurons were also decreased by the supernatant of macrophages in vincristine (CM_{M0+VCR} , $74.27 \pm 2.42 \mu m$ vs $100.08 \pm 2.73 \mu m$, $p < 0.001$, $n = 6$) and LPS (CM_{M1} , $59.97 \pm 2.93 \mu m$ vs $100.08 \pm 2.73 \mu m$, $p < 0.001$, $n = 6$) (Figure 3C, D). The number of neurons and axonal length decreased more in the vincristine and LPS combined stimulation group (CM_{M1+VCR}) than in the LPS alone stimulation group (CM_{M1}) (7.43 ± 0.82 vs 10.60 ± 0.37 ; $49.38 \pm 2.33 \mu m$ vs $59.97 \pm 2.93 \mu m$, $p < 0.05$, $n = 6$, Figure 3C, D). These results were consistent with the increase of proinflammatory factors released by RAW264.7 cells following treatment of vincristine and LPS. This led us to further suspect that the colonic myenteric neurons injury in system treatment of vincristine might be probably caused by macrophage polarization and the released proinflammatory factors.

Macrophages depletion alleviated colonic myenteric neurons injury and decreased proinflammatory factors

To further confirm that the colonic myenteric neurons injury was caused by colonic macrophages in system treatment of vincristine, we used the clodronate liposomes (200 μL /mice, i.p.) to deplete peripheral macrophages. This macrophage depletion method had been proven to be reliable and widely used in many models [41, 46]. We also proved that the macrophage depletion efficiency of the liposome (Supplemental Figure 3). The results showed that there were no significant differences in the number and length of colonic neurons between the Clodronate Liposomes group and the Control Liposomes group, which proved that the solvent had no side effects on the mice. However, compared with Control Liposomes + VCR group, in Clodronate Liposomes + VCR group the number (52.44 ± 3.83 vs 39.55 ± 1.97 , $p < 0.01$, $n = 4$, Figure 4B) and the length of axons ($94.43 \pm 1.03 \mu m$ vs $63.60 \pm 2.54 \mu m$, $p < 0.001$, $n = 4$, Figure 4C) of colonic neurons were significantly increased. At the same time, we detected the changes of proinflammatory factor expression in LMMP of colon. Compared with Control Liposomes + VCR group, in Clodronate Liposomes + VCR group IL-1 β , IL-6, TNF- α in the expression of mRNA decreased (4.35 ± 0.97 times vs 14.78 ± 2.49 times, 2.77 ± 0.76 times vs 11.94 ± 1.77 times, 1.63 ± 0.35 times vs 8.03 ± 1.07 times, $p < 0.01$, $n = 6$) and protein levels had the same trend (Figure 4D-F). However, compared with the Control Liposomes group, mRNA expression of Arg1 and IL-10 decreased in the other three groups and no significant difference between the three groups (Supplemental Figure 4 A, B). The results suggested that macrophage depletion reduced the increase of proinflammatory factors caused by vincristine and alleviated the colonic myenteric neurons injury.

Macrophages depletion alleviated colonic myenteric neurons injury *in vivo*

In addition to examining the colonic myenteric neurons injury, we also detected the changes of neurons in colon slices by immunofluorescence (Figure 5A). Compared with the Control Liposomes group, in Control Liposomes + VCR group, the number of neurons decreased in every $10^4 \mu m^2$ ganglion region (42.46 ± 4.64 vs 66.52 ± 3.37 , $p < 0.01$, $n = 6$, Figure 5B). Compared with Control Liposomes + VCR group, in Clodronate Liposomes + VCR group the number of neurons was significantly restored (55.54 ± 4.15 vs 42.46 ± 4.64 , $p < 0.05$, $n = 6$, Figure 5B). Compared with Control Liposomes + VCR group, in Clodronate Liposomes + VCR group the total gastrointestinal transport time decreased (168.38 ± 10.34 min vs

197.88 ± 13.04 min, $p < 0.05$, $n = 7$, Figure 5C). These findings suggested that in system treatment of vincristine the colonic myenteric neurons injury and enteric transport was associated with M1-type macrophages.

Vincristine stimulated M1 macrophages polarization through increasing phosphorylation of ERK1/2 and p38-MAPK

ERK1/2 and p38-MAPK, members of the MAPKs family, are stimulated by various extracellular factors to regulate various biological processes, including inflammatory response [47]. They are important transmitter of signals from the cell surface to nucleus. It has been reported that LPS stimulation increased phosphorylation of MAPKs [48]. In our study, we used RAW264.7 cells to investigate the effect of vincristine on phosphorylation of these two proteins by western blot (Figure 6A). Compared with the control group, RAW264.7 cells stimulated by vincristine increased the phosphorylation of p44/42 and p38 (1.27 ± 0.07 vs 0.69 ± 0.13 , 0.92 ± 0.43 vs 0.50 ± 0.04 , $p < 0.001$, $n = 6$, Figure 6B, C). Compared with the control group, RAW264.7 cells stimulated by LPS in combination with VCR increased the phosphorylation of p44/42 and p38 (1.62 ± 0.09 vs 1.31 ± 0.12 , 1.13 ± 0.04 vs 0.94 ± 0.03 , $p < 0.01$, $n = 6$, Figure 6B, C). Therefore, our results showed that vincristine regulated M1-type macrophages polarization by stimulating the ERK1/2 and p38-MAPK signaling pathways.

Proinflammatory factors caused colonic myenteric neurons injury via the SGK1-FOXO3 pathway

In order to investigate the mechanism underlying the effect of proinflammation factors on colonic myenteric neurons injury, we performed RNA-seq analysis of LMMP with or without depleted macrophages in system treatment of vincristine. The results of heat map and volcanic map showed that in Clodronate Liposomes + VCR group, 408 genes were up-regulated and 180 genes were down-regulated compared with Control Liposomes + VCR group (Figure 7A, B). From these differential genes we identified two genes regulating cell survival, SGK1 and FOXO3. SGK1 is a homologue of AKT, and the regulated protein is an important anti-apoptotic kinase [49, 50]. SGK1 regulates the downstream target FOXO3 protein phosphorylating and suppresses FOXO3 mediated transcription [50]. We first verified these two genes at the mRNA level by qRT-PCR. Compared with the Control Liposomes group, the mRNA level of SGK1 in the Control Liposomes + VCR group decreased to 0.53 ± 0.13 times ($p < 0.05$, $n = 4$, Figure 7C). Compared with the Control Liposomes + VCR group, the SGK1 mRNA level was significantly increased in Clodronate Liposomes + VCR group (1.13 ± 0.22 times vs 0.53 ± 0.13 times, $p < 0.05$, $n = 4$, Figure 7C). The expression of FOXO3 at mRNA level showed the same trend as the SGK1 (Figure 7D). The verification results of qRT-PCR were consistent with those of RNA-seq. Then we analyzed the protein and protein phosphorylation level expression of these two proteins by western blot (Figure 7E). Compared with the Control Liposomes group, SGK1 and p-SGK1 levels were decreased in Control Liposomes + VCR group (0.39 ± 0.04 vs 0.88 ± 0.08 , $p < 0.001$, 0.51 ± 0.11 vs 1.01 ± 0.10 , $p < 0.01$, $n = 6$, Figure 7F, G). Compared with the Control Liposomes + VCR group, in Clodronate Liposomes + VCR group, the SGK1 and p-SGK1 levels were significantly increased (0.81 ± 0.06 vs 0.39 ± 0.04 , $p < 0.001$, 0.94 ± 0.07 vs 0.51 ± 0.11 , $p < 0.01$, $n = 6$, Figure 7F, G). P-FOXO3/FOXO3 level was decreased in Control Liposomes + VCR group compared with

the Control Liposomes group (0.75 ± 0.03 vs 1.00 ± 0.03 , $p < 0.001$, $n = 6$, Figure 7H). In Clodronate Liposomes + VCR group, the p-FOXO3/FOXO3 levels were increased compared with Control Liposomes + VCR group (1.07 ± 0.03 vs 0.75 ± 0.03 , $p < 0.001$, $n = 6$, Figure 7H). These results suggested that in system treatment of vincristine, the apoptosis of colonic myenteric neuron was through decreasing the expression of SGK1 and p-SGK1 and decreasing the level of p-FOXO3/FOXO3.

Proinflammatory factors increased apoptosis of colonic myenteric neurons

It has been reported that there is constant apoptosis and renewal in mature intestinal neurons [51]. And because the SGK1/FOXO3 pathway promotes cell survival by inhibiting apoptosis, we measured the number of TUNEL⁺ myenteric neurons (Figure 8A). Compared with the Control Liposomes group, in Control Liposomes + VCR group, the number of TUNEL⁺ neurons in every 10^4 um^2 ganglion region was significantly increased (25.19 ± 2.79 vs 8.63 ± 2.04 , $p < 0.001$, $n = 6$, Figure 8B). Compared with the Control Liposomes + VCR group, in Clodronate Liposomes + VCR group, the number of TUNEL⁺ neurons in every 10^4 um^2 ganglion region decreased significantly (15.17 ± 3.17 vs 25.19 ± 2.79 , $p < 0.01$, $n = 6$, Figure 8B). We also detected the expression level of cleaved caspase-3 by western blot (Figure 8C). Compared with the Control Liposomes group, in Control Liposomes + VCR group, cleaved caspase-3/GAPDH was significantly increased (0.81 ± 0.06 vs 0.42 ± 0.02 , $p < 0.001$, $n = 6$, Figure 8D). Compared with the Control Liposomes + VCR group, in Clodronate Liposomes + VCR group, cleaved caspase-3/GAPDH decreased significantly (0.57 ± 0.08 vs 0.81 ± 0.06 , $p < 0.01$, $n = 6$, Figure 8D). This results further proved that the proinflammatory factors led to colonic myenteric neurons apoptosis, which was alleviated after depletion of macrophages.

Discussion

Our current study demonstrated that system treatment with vincristine induced colonic myenteric neurons injury. The injury was mainly manifested in the reduction of the number of neurons and the shortening of the length of axons. The main cause of the injury was targeted on the colonic macrophages. Vincristine increased M1-type macrophages polarization with increased expression of proinflammatory factors, including IL-1 β , IL-6 and TNF- α . These proinflammation factors induced the apoptosis of colonic myenteric neurons. Depletion of macrophages alleviated this injury. The proinflammatory effect of vincristine functioned mainly by strengthening the phosphorylation of ERK1/2 and p38-MAPK in macrophages (Figure 9). These proinflammatory factors induced colonic myenteric neurons injury via SGK1-FOXO3 pathway (Figure 9). For the first time, we proved that vincristine was directly involved in the modulation of M1-type macrophages polarization in colon. It was also the first time to demonstrate that system treatment of vincristine induced colonic myenteric neurons injury caused by M1 macrophages. The regulation of macrophages polarization might be a new target to alleviate the colonic myenteric neurons injury caused by system treatment of vincristine.

Our results firstly proved that after system treatment with vincristine, colonic myenteric neurons were injured. This reminded us of the peripherally neurotoxic effects of vincristine. The clinical application of

vincristine is accompanied by serious side effects, and peripheral neurotoxicity accounts for 50%-90% of chemotherapy patients [52], which is one of the important reasons for patients to stop chemotherapy. After systematically treated with vincristine, up to 57% of patients have digestive system symptoms such as constipation and abdominal pain [22, 23]. Therefore, the need for new and effective method to mitigate peripheral neurotoxicity is urgent. As there was mounting evidence proving that neurological injury is associated with neuroinflammation, we detected the changes in inflammation after system treatment with vincristine. We found that after system treatment with vincristine, the expression of proinflammation factors increased not only in mRNA level but also in protein level. It is worth noticing that the total gastrointestinal transport time was lengthened by system treatment of vincristine. These findings also supported the role of vincristine in the pathogenesis of constipation after chemotherapy. This suggested us that colonic myenteric neurons injury might be related to an increase of proinflammatory factors.

Enteric macrophages had been shown to be responsible for driving inflammation and damaging gastrointestinal neurons and demonstrated in postoperative intestinal obstruction, enteritis, senescence, intestinal ischemia-reperfusion injury and other models [53-55]. In recent years, more and more studies have reported that macrophages in the intestine are similar to microglia in the central nervous system [56, 57]. Vincristine could activate microglia in central nervous system, leading to the release of proinflammatory factors and neuroinflammation [14]. We treated RAW264.7 cells with vincristine *in vitro*, obtained similar results with experiments *in vivo*. Vincristine stimulated macrophage polarization toward M1-type to produce proinflammatory factors IL-1 β , IL-6 and TNF- α . When vincristine combined with LPS, macrophages produced more proinflammatory factors. These results suggested us that vincristine stimulated M1-type macrophage polarization, which might have a pathway that intersects with LPS. It has been reported that macrophages stimulated by LPS could increase phosphorylation of MAPKs [48]. MAPKs as a serine-threonine protein kinase family, including p38-MAPK, ERK1/2 and JNK, can be activated by different extracellular stimuli, such as cellular stress, hormones, cytokines and neurotransmitters to regulate basic biological processes [47]. Our results showed that vincristine stimulated macrophages to increase phosphorylation of p38-MAPK and ERK1/2. Moreover, when macrophages were stimulated by vincristine combined with LPS, phosphorylation of p38-MAPK and ERK1/2 were enhanced more. This results suggested that *in vitro* vincristine co-activated this pathway in synergized with LPS to stimulate M1 macrophage polarization.

In order to better simulate the colonic myenteric neurons injury caused by these proinflammatory factors *in vitro*, colonic myenteric neurons were incubated in different media. We found that the number of neurons and the length of axons decreased when cultured in the medium of macrophages stimulated by vincristine. The number of neurons and the length of axons were further decreased when cultured in the medium of macrophages stimulated by vincristine in combined with LPS. These changes of neurons cultured in conditioned medium might be attributed to the proinflammatory factors produced by macrophages stimulated by vincristine *in vitro*. These results once again demonstrated the important role of proinflammatory factors in enteric myenteric neurons injury.

In addition to macrophages, other cells might also be involved in the observed inflammation. To further determine the role of macrophages in neurons injury following system treatment of vincristine, we conducted a direct verification through *in vivo* macrophage depletion. We specifically depleted macrophages of mice by the clodronate liposomes, this method had been proven to be reliable and widely used in stroke models [46] and Parkinson's disease [41]. Our results showed that continuous macrophage depletion significantly reduced colonic proinflammatory factors and mitigated colonic myenteric neurons injury. This result proved that the proinflammatory factors produced by the macrophage stimulated by vincristine played a major role in enteric myenteric neurons injury *in vivo*. Although macrophage depletion was not suitable for clinical application, it provided a more direct method to study the role of macrophages in systematically treated with vincristine.

In order to demonstrate how these proinflammatory factors induced colonic myenteric neurons injury, mRNA sequencing was performed on the LMMP in mice with system treatment of vincristine. It was found that the two important mRNA SGK1 and FOXO3 in the depleted macrophages vincristine treatment group were up-regulated compared with the non-depleted macrophages vincristine treatment group. Serum and glucocorticoid-induced kinase 1 (SGK1) is regulated by a variety of cellular stress stimuli and participates in the regulation of neuronal excitability, inflammation, proliferation and apoptosis [50]. It is a strong anti-apoptotic kinase. SGK1 is an Akt homologue, and has 50-55% protein sequence homology with Akt [49, 50]. However, there are also differences between them. Compared with Akt, SGK1 lacks the homologous domain targeting Akt to cell membrane [50], so it's easier to get into the cytoplasm and the nuclear sites. Previous studies have shown that overexpression of SGK1 can reduce apoptosis damage of renal cells due to hypoxia/re-oxygenation [58]. We showed for the first time that the expression of SGK1 and p-SGK1 decreased in LMMP of mice system treatment of vincristine. Previous studies demonstrated that SGK1 knockdown aggravates H/R-induced cells damage [59, 60]. The Forkhead-like transcription factor O3 (FOXO3) is the most characteristic downstream target of SGK1 and SGK1 regulates FOXO3 phosphorylating [49, 58]. Phosphorylation of FOXO3 promotes FOXO3 withdrawal from the nucleus into the cytoplasm, thereby inhibiting transcription and promoting cell survival [61]. We further verified the phosphorylation level of FOXO3. We found that in LMMP of mice system treatment with vincristine the FOXO3 phosphorylation decreased. We also observed the apoptosis of colonic myenteric neurons via detecting the TUNEL⁺ neurons and cleaved caspase-3. After system treatment with vincristine, the apoptosis of colonic myenteric neurons increased. This result was consistent with the change of SGK1 and FOXO3 expression levels, which demonstrated that proinflammatory factors induced colonic myenteric neurons apoptosis in an SGK1/FOXO3 dependent manner.

Conclusion

In summary, the current findings demonstrated that colonic myenteric neurons injury caused by system treatment of vincristine was induced by regulated the polarization of colonic macrophages to M1-type through p38-MAPK and ERK1/2 pathway. These proinflammatory factors induced colonic myenteric neurons apoptosis in an SGK1/FOXO3 dependent manner. Our study provides a new mechanism

underlying the enteric neurons injury following the system treatment of vincristine and a potential target for the mitigation of the side effects of vincristine in clinical practice.

Abbreviations

ENS, enteric nervous system

LMMP, longitudinal muscle myenteric plexus

VCR, vincristine

DMEM, Dulbecco's Modified Eagle's Medium

MMs, muscularis macrophages

CD86, cluster of differentiation 86

LPS, lipopolysaccharide

IL, interleukin

TNF- α , tumor necrosis factor- α

Arg1, arginase1

ERK, extracellular signal-related kinase

JNK, c-Jun N-terminal kinase

MAPK, mitogen-activated protein kinase

SGK1, serum and glucocorticoid-induced kinase 1

FOXO3, Forkhead Box O3

DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride hydrate

FBS, fetal bovine serum

qRT-PCR, quantitative reverse transcription–polymerase chain reaction

Declarations

- Ethical Approval and Consent to participate

We conducted animal experiments according to International Guiding Principle for

Animal Research that were stipulated by Council for International Organizations of Medical Sciences (CIOMS), the routines have obtained approval from Shandong University Animal Ethics Committee. The participants working with animal model received training abiding by the rules of Institutional Animal Care and Use Committee Guidebook (IACUC). And written informed consent was obtained from all participants.

- Consent for publication

Not applicable. This article does not contain any individual person's data.

- Availability of supporting data

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

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-Autors contribution

CYL: designed studies, analyzed data and revised the manuscript; YFG: performed the majority of the laboratory work, generated the final figures and wrote the manuscript; YT: performed animal model; HJZ: performed motor function testing; XLC: performed flow cytometry; BY and JXL: manuscript proof reading and editing;

All authors read and approved the final draft of this manuscript.

-Competing interests

The authors declare that there are no competing interests associated with the manuscript.

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Figures

Figure 1

Vincristine resulted in colonic myenteric neurons injury and proinflammatory factors increase At the end of the 10-day system treatment of vincristine, (A) colonic myenteric neurons were cultured for 6 days in vitro for immunofluorescence staining with representative images of β III-Tubulin (green) and DAPI (blue).

Scale bar = 50 μ m. N = 6/group. (B, C) Quantitative analyses of number of neurons and length of axons. Six randomly images were captured of per animal. N = 6/group. (D) Total gastrointestinal transport time was measured in both groups. (E, F) Representative flow cytometric CD206+ cells average percentage. N = 7/group. (G, H) Representative flow cytometric CD86+ cells average percentage. N = 7/group. (I, J) mRNA or protein levels of proinflammatory factors IL-1 β , IL-6, and TNF- α were detected by qRT-PCR or ELISA. N = 6/group. Values are mean $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 according to t-test.

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Figure 2

Vincristine promoted the M1-type polarization of RAW264.7 cells (A, B) Vincristine (from 10^{-10} M to 10^{-8} M) stimulated RAW264.7 to increase the transcription level of IL-1 β and IL-6. N = 6/group. (C, D) Vincristine (from 10^{-10} M to 10^{-8} M) in coordination with LPS (100 ng/ml) stimulated RAW264.7 to increase the mRNA level of IL-1 β and IL-6. N = 6/group. (E) The mRNA levels of Arg1 and IL-10 were detected through vincristine or vincristine in coordination with IL-4 activating RAW264.7 by qRT-PCR. N = 6/group. (F-H) The mRNA and protein levels of IL-1 β , IL-6 and TNF- α were detected through vincristine or vincristine in coordination with LPS activating RAW264.7 by qRT-PCR (N = 6/group) or ELISA (N = 4/group). Values are mean $\pm$ SEM, *p < 0.05, **p < 0.01, ***p < 0.001 according to ANOVA with Newman-Keuls.

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Figure 3

Supernatant of vincristine stimulated peritoneal macrophages induced colonic myenteric neurons injury in vitro (A) The culture procedure of colonic myenteric plexus neurons using conditioned medium. (B) Representative photographs of immunofluorescent staining of β III-Tubulin (green) and DAPI (blue). Scale bar = 50 μ m. N = 6/group. (C, D) Quantitative analyses of number of neurons and length of axons. Six

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Figure 4

Macrophages depletion alleviated colonic myenteric neurons injury and decreased proinflammatory factors (A) Representative photographs of immunofluorescent staining of β -Tubulin (green) and DAPI (blue) among four groups. Scale bar = 50 μ m. N = 4/group. (B, C) Quantitative analyses of number of neurons and length of axons. Six randomly images were captured of per animal. N = 4/group. (D-F) The mRNA and protein levels of IL-1 β , IL-6 and TNF- α were detected among these four groups by qRT-PCR or ELISA. N = 6/group. Values are mean $\pm$ SEM, * p < 0.05, ** p < 0.01, *** p < 0.001 according to ANOVA with Newman-Keuls.

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Figure 5

Macrophages depletion alleviated colonic myenteric neurons injury in vivo (A) Representative photographs of immunofluorescent staining of β -Tubulin (red) and DAPI (blue) among four groups. Scale bar = 50 μ m. N = 4/group. (B) Quantitative analyses of the number of neurons in every 104 μ m² ganglion region. Six randomly images were captured of per animal. N = 4/group. (C) Total gastrointestinal transport time was measured in four groups. N = 7/group. Values are mean $\pm$ SEM, *p < 0.05, **p < 0.01, ***p < 0.001 according to ANOVA with Newman–Keuls.

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Figure 6

Vincristine stimulated M1 macrophages polarization through increasing phosphorylation of ERK1/2 and p38-MAPK (A) RAW264.7 were treated with vincristine (10–9M) or in coordination with LPS (100 ng/ml) for 2h. Representative immunoblots are detected for phosphorylated p44/42 (p-p44/42), total p44/42, phosphorylated p38 (p-p38), and total p38 with western blot analysis. (B, C) Quantitative analyses of the relative levels of p-p44/42 / p44/42, p-p38 / p38 within each group. N = 6/group. Values were normalized to GAPDH. Values are mean $\pm$ SEM, *p < 0.05, **p < 0.01, ***p < 0.001 according to ANOVA with Newman–Keuls.

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Figure 7

Proinflammatory factors caused colonic myenteric neurons injury via the SGK1-FOXO3 pathway. In system treatment of vincristine, RNA-seq analysis of two groups of LMMP with or without depleted macrophages. The results of heat map (A) and volcanic map (B) showed the differential genes. N = 3/group. (C, D) SGK1 and FOXO3 at the mRNA level by qRT-PCR. N = 6/group. (E) Representative immunoblots are detected for phosphorylated SGK1 (p-SGK1), total SGK1, phosphorylated FOXO3 (p-

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Figure 8

Proinflammatory factors increased apoptosis of colonic myenteric neurons (A) Representative photographs of immunofluorescent staining of TUNEL (green), β -Tubulin (red) and DAPI (blue). Scale bar = 50 μ m. N = 6/group. (B) Quantitative analyses of the number of neurons and TUNEL+ neurons in every 104 μ m² ganglion region. Six randomly images were captured of per animal. N = 6/group. (C) Representative immunoblots are detected for cleaved caspase-3 with western blot analysis in each group. N = 6/group. Values were normalized to GAPDH. (D) Quantitative analyses of the relative levels of cleaved caspase-3/GAPDH within each group. N = 6/group. Values are mean $\pm$ SEM, *p < 0.05, **p < 0.01, ***p < 0.001 according to ANOVA with Newman–Keuls.

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Figure 9

Proposed mechanisms for apoptosis of colonic myenteric neurons in system treatment with vincristine. Vincristine firstly regulated the polarization of colonic macrophages to M1-type through p38-MAPK and ERK1/2 pathway, and produced proinflammatory factors. These proinflammatory factors inhibited SGK1 expression and phosphorylation of FOXO3 and in turn led to an increase of cleaved caspase-3.

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