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Resveratrol alleviates oxidative damage in enteric neurons and associated gastrointestinal dysfunction caused by chemotherapeutic agent oxaliplatin

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ABSTRACT

Oxaliplatin is a first-line chemotherapeutic agent used for the treatment of colorectal cancer. Its use is associated with severe gastrointestinal (GI) side-effects, associated with oxidative damage and neurotoxicity to the enteric neurons. Resveratrol is a potent anti-oxidant that has shown to exert protection against oxidative damage and neurotoxicity in other neurons and could therefore prevent oxaliplatin-induced damage to enteric neurons. We determined whether co-administration of resveratrol with oxaliplatin alleviates enteric neuron toxicity and GI dysfunction in mice. Colons were collected for immunohistochemical analysis of myenteric neurons and assessment of motor activity in organ-bath experiments. Morphological damage to the colonic mucosa and muscles was analysed. Oxaliplatin treatment induced translocation of nitrated proteins into the nuclei of myenteric neurons and significant damage to the mucosal lining, vacuolisation and a decrease in muscle thickness. This damage is linked to motor dysfunction due to inhibition of the amplitude of colonic contractions leading to chronic constipation. Co-treatment with resveratrol prevented oxaliplatin-induced neurotoxicity, alleviated damage to GI mucosa, crypts and muscle layer resulting in improved contractility and a decrease in constipation resveratrol could be integrated as part of a therapeutic regimen to help alleviate oxaliplatin-induced GI dysfunction.

Key words:

Colorectal cancer

Resveratrol

Oxaliplatin

Neurotoxicity

Enteric neurons

1. Introduction

Colorectal cancer (CRC) has the highest incidence rate and the second highest mortality rate of all cancers worldwide [1, 2]. The first line of chemotherapeutic treatment for advanced CRC consists of the platinum-based compound oxaliplatin in combination with 5-Fluorouracil and Leucovorin [3]. Although very effective in the treatment of CRC, there are many adverse effects associated with oxaliplatin treatment. The most severe and dose limiting properties of oxaliplatin treatment are the gastrointestinal (GI) side-effects such as

nausea, vomiting, diarrhoea and peripheral sensory neuropathy [4, 5]. These side-effects can persist for years even after treatment has ceased [6]. Dose limitations and in some instances, cessation of treatment, are often as a result of these side-effects. It is imperative that new treatment approaches are developed to prevent side-effects and increase the cytotoxic efficacy of treatment. The mechanisms by which these side-effects occur have been of interest lately. Recent findings suggest that oxidative damage and toxicity to the enteric neurons innervating the GI tract and controlling its functions may contribute to long-term side-effects of oxaliplatin treatment [7-9]. Thus, developing treatments that prevent oxidative damage to the enteric neurons is a viable pathway for improving patient quality of life.

Nitric oxide (NO) is produced by the activation of nitric oxide synthase (NOS), and is a widely distributed neurotransmitter located within both the central and peripheral nervous systems. NO has various location-dependant functions, and, in the GI tract acts as a mediator of vasodilation and GI relaxation [10, 11]. In the enteric neurons embedded in the GI tract wall, neuronal NOS (nNOS) is expressed by descending interneurons and inhibitory motor neurons supplying the intestinal smooth muscle [12]. Release of NO from neurons and smooth muscle is essential for the complex muscle co-ordinations that produce GI peristaltic motility [13, 14]. NO can become toxic in a reaction with superoxide to form peroxynitrite. Peroxynitrites are reactive nitrogen species (RNS) that have the ability to modify tyrosine residues in proteins to create nitrotyrosines. Nitration of structural proteins has been shown to mediate oxidative damage to neurons and cause pathological complications [15]. The presence of RNS 3-nitrotyrosine (3-NT) in the nucleus of neuronal cells is an indicator of oxidative damage and neurotoxicity, and is associated with neuronal death [16].

Resveratrol (3,5,4'-trihydroxy-stilbene) is a potent polyphenol that is found naturally in grapes, plums, and peanuts [17]. There have been many studies reporting anti-oxidant, anti-inflammatory [18, 19] neuroprotective [20-22] and anti-cancer [23-26] effects of resveratrol. Studies investigating the anti-oxidant capabilities of resveratrol have demonstrated that it is a powerful scavenger of free radicals, can modulate and enhance cellular anti-oxidant defence mechanisms and has the ability to stimulate the synthesis of anti-oxidant enzymes [27, 28]. Additionally, resveratrol has been found to function synergistically with chemotherapeutic compounds to increase cytotoxic efficacy of anti-cancer treatments *in vivo* and *in vitro* [29-33]. To date, no studies have investigated the ability of resveratrol to alleviate oxaliplatin-induced oxidative damage of the enteric neurons associated with GI side-effects.

2. Methods

2.1. Animals and Ethical Approval

Male BALB/c mice aged 5-8 weeks (18 – 25g) were used in this study. Mice were supplied from the Animal Resources Centre (Perth, Australia). Animals were housed in groups of 3-5 and were kept in an animal holding room with a 12-hour light and dark cycle at approximately 22 °C with free access to food and water. The mice were allowed to acclimatise for at least one week before receiving injections. All experimental work in this study was approved by the Victoria University Animal Experimentation Ethics Committee and performed in accordance with the guidelines of the Australian National Health and Medical Research Council.

2.2. Oxaliplatin Treatment

Mice received intraperitoneal (i.p.) injections of oxaliplatin (Tocris Bioscience, UK) 3 mg/kg/dose 3 times a week with a 26 gauge needle for 2 weeks as previously reported [8]. Oxaliplatin was dissolved in sterile water in order to make 10^{-2} M stock solutions and refrigerated at -20°C . The stock was then defrosted and diluted further to make 10^{-3} mM solutions for intraperitoneal injections. The dose of oxaliplatin was calculated to be equivalent to standard human dose per body surface area [34, 35]. Vehicle-treated mice received sterile water via i.p. injections 3 times a week with a 26 gauge needle. Maximum volume did not exceed 200 μL per injection. Mice were euthanized via cervical dislocation 14 days after the first oxaliplatin injection and colon tissues were collected for *in vitro* experiments.

2.3. Resveratrol Treatment

Mice received i.p. injections of resveratrol (Sigma-Aldrich, Australia) at a 500 $\mu\text{g}/\text{kg}/\text{dose}$ daily with a 26 gauge needle for 3 weeks. Resveratrol was dissolved in 100 % ethanol (EtOH) as per directions from the supplier. Daily resveratrol injections were made fresh each day as a 25 % dilution in sterile water. The resveratrol + oxaliplatin-treated group received the same dose of resveratrol injected daily 7 days prior to and every day during

oxaliplatin treatment. Maximum volume did not exceed 200 μ L per injection. Vehicle-treated group received 25% EtOH+sterile water injected daily with a 26 gauge needle. The cumulative volume for mice receiving 2 injections in one day (resveratrol + oxaliplatin or 25 % EtOH + sterile water) did not exceed 400 μ l. Oxaliplatin was administered 15 minutes (min) after resveratrol. Mice were euthanized via cervical dislocation 21 days after the first injection. Colon tissues were collected for *in vitro* experiments.

2.4. Colonic Motility Assessment

The entire colon was removed from treated mice and set up in organ-bath chambers to record motor patterns *ex vivo* as described previously [9]. Briefly, the colon was placed into warmed (35 °C), oxygenated physiological saline (composition in mM: NaCl 118, KCl 4.6, CaCl₂ 3.5, MgSO₄ 1.2, NaH₂PO₄ 1, NaHCO₃ 25, d-Glucose 11; bubbled with 95%O₂ and 5% CO₂) until the faecal pellets were expelled. The empty colon was cannulated at both ends and arranged horizontally in organ-bath chambers. The proximal end of the colon was connected to a reservoir containing oxygenated physiological saline to maintain a baseline intraluminal pressure. The distal end was attached to an outflow tube that provided a maximum of 2 cm H₂O back-pressure. Organ baths were continuously perfused with oxygenated physiological saline solution and preparations were left to equilibrate for 30 min. Contractile activity of each segment was recorded with a Logitech Quickcam Pro camera positioned 7–8 cm above the preparation. Videos (4x15min) of each test condition were captured and saved in *avi* format using VirtualDub software (version 1.9.11).

Recordings were used to construct spatiotemporal maps using in-house edge detection software [36]. Spatiotemporal maps plot the diameter of the colon at all points during the recording allowing contractile motor patterns to be analysed with MatLab software (version 12).

2.5. Immunohistochemistry

Segments of the colon were placed in oxygenated phosphate-buffered saline (PBS) (pH 7.2) containing nifedipine (3 μ M) (Sigma-Aldrich, Australia) for 20 min to inhibit smooth muscle contraction. Samples were cut open along the mesenteric border, cleared of their contents, maximally stretched and dissected to expose the myenteric plexus. Mucosa,

submucosa and circular muscle layers were removed and tissues containing longitudinal muscle and myenteric plexus were then fixed with Zamboni's fixative (2 % formaldehyde, 0.2 % picric acid) overnight at 4 °C. Preparations were cleared of fixative by washing 3 times for 10 min with Dimethyl Sulfoxide (DMSO) (Sigma-Aldrich, Australia) followed by 3 x 10 min washes with PBS. Fixed tissues were stored at 4°C in PBS for a maximum of 5 days. Wholemount preparations were incubated with 10% normal donkey serum (Chemicon, USA) for 1 hour at room temperature. Tissues were then washed (2 x 5 mins) with PBS and incubated with a primary antibody against 3-Nitrotyrosine (3-NT) (rabbit, 1:1000, Millipore, CA, USA) overnight at 4 °C. Tissues were then washed 3 x 10 min in PBS before incubation with species-specific secondary antibody donkey anti-rabbit 488 (1:200, Jackson ImmunoResearch Laboratories, PA, USA) for 2 hours at room temperature. Wholemount preparations were given 3 final 10 min washes in PBS and then mounted to glass slides using fluorescent mounting medium (DAKO, Australia).

2.6. Neuronal Cell Counting and Imaging

Wholemount preparations were observed as three dimensional (z-series) images under a Nikon Eclipse Ti laser scanning confocal microscope (Nikon, Japan), 8 randomly chosen images from each preparation were captured with a 20x objective and processed using NIS Elements software (Nikon, Japan). The number of neurons displaying translocation of nitrated proteins to the nuclei was quantified in myenteric ganglia within a 2mm² area of each distal colon preparation. Fluorophores were visualized using excitation filters for Alexa 488 (excitation wavelength 473nm). Z-series images were taken at step size of 1.75µm (1600 x 1200 pixels).

2.7. Histology

The distal colon was harvested and placed in a 10 % formalin solution overnight and transferred into 70 % ethanol the following day. Paraffin-embedded colon sections were cut into 5 µm thick sections and de-waxed in a 60 °C oven for 30 min. To examine the morphological changes to the colon, a standard Hematoxylin and Eosin staining protocol was followed [37]. Ten sections per preparation were analysed. All images were analyzed blindly.

2.8. Data and Statistical Analysis

Sample size was calculated based on our previous studies on enteric neuropathy and intestinal dysmotility associated with chemotherapy [8, 38]. To detect a 30% change at a power 0.8 and $\alpha = 0.05$ with 10% SD, the effect size should be minimum $n=5$ animals per group as calculated by the GPOWER program. Data were assessed using one way ANOVA and Tukey's post-hoc test. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., CA, USA). Data are presented as mean \pm standard error of the mean (SEM). Value differences were considered statistically significant at $P<0.05$.

3. Results

3.1. Treatment with resveratrol prevented oxaliplatin-induced translocation of nitrated proteins into the nucleus of myenteric neurons

Repeated oxaliplatin administration in mice was associated with a significant ($***P<0.001$) increase in the translocation of nitrated proteins into the nuclei of myenteric neurons (38 ± 1 neurons/ 2mm^2) when compared to vehicle-treated mice (17 ± 1 neurons/ 2mm^2) (Figure 1A-B). Resveratrol administered in combination with oxaliplatin prevented oxaliplatin-induced translocation of nitrated proteins into neuronal nuclei (17 ± 1 neurons/ 2mm^2). Resveratrol treatment alone had no effect on the amount of translocation (12 ± 1 neurons/ 2mm^2) similar to the vehicle group.

3.2. Treatment with resveratrol prevented oxaliplatin-induced mucosal damage and loss of smooth muscle in the distal colon

Gross morphological assessment of distal colon segments from treated and untreated mice showed significant changes had occurred. Oxaliplatin-treated mice demonstrated significant damage to the mucosal lining, vacuolisation, and a decrease in muscle thickness ($73\pm 17\mu\text{m}$; $***P<0.001$) when compared to vehicle ($240\pm 5\mu\text{m}$) and resveratrol-treated mice ($243\pm 12\mu\text{m}$) (Figure 1C-D). Resveratrol + oxaliplatin treatment alleviated damage to the mucosa and muscle layer width ($153\pm 20\mu\text{m}$) but was not comparable to vehicle-treated

mice, with a decrease in muscle width ($*P<0.05$), but was significantly different to oxaliplatin-treated group.

3.3. Treatment with resveratrol alleviated oxaliplatin-induced changes to colonic motor activity

Oxaliplatin-treated mice demonstrated reduction in the amplitude of colonic contractility, with the change in diameter ($48\pm 13\text{mm}$) significantly less ($***P<0.001$) than that observed in vehicle-treated mice ($129\pm 9\text{mm}$) (Figure 2A-B). Administration of resveratrol in conjunction with oxaliplatin alleviated the reduction in the amplitude of contractions and the change in diameter between relaxation and contraction was not significantly different to vehicle-treated group ($117\pm 8\text{mm}$). Resveratrol treatment alone had no effect on the change in diameter of the distal colon ($142\pm 4\text{mm}$).

3.4. Treatment with resveratrol alleviated oxaliplatin-induced constipation

The number of pellets present in the colon of mice after culling is an indication of colonic function. Chronic oxaliplatin-treatment caused significant constipation (6 ± 0.3 pellets/colon, $***P<0.001$) when compared to vehicle (1.7 ± 0.2 pellets/colon) and resveratrol (1.8 ± 0.3 pellets/colon) treatments (Figure 2C). Resveratrol in combination with oxaliplatin was able to partially alleviate this (3.3 ± 0.3 pellets/colon), however the level of constipation was still increased ($*P<0.05$) when compared to vehicle-treated mice.

4. Discussion

Here we present one of the first studies to demonstrate that resveratrol administered in conjunction with oxaliplatin prevents oxidative damage and neurotoxicity as well as alleviating colonic dysfunction and constipation.

4.1. Resveratrol alleviates oxaliplatin-induced oxidative damage in enteric neurons

We used the marker anti-3-NT antibody to identify the translocation of nitrated proteins in neuronal nuclei as an indicator of oxidative damage and neurotoxicity.

Translocation occurred most significantly in the enteric neurons of oxaliplatin-treated mice, and was reduced to the level of vehicle-treated group in the resveratrol + oxaliplatin-treated animals. Previous studies have shown translocation of nitrotyrosine in myenteric neurons following ischemia and reperfusion in mice [39] and induction of colitis in rats [40]. Accumulation and translocation of nitrotyrosine has been linked to protein misfolding, mitochondrial dysfunction, neuronal degeneration [41] and an increased susceptibility to NO-induced apoptosis [42]. The results of this study are in agreement with previously reported increased level of superoxide production, cytochrome *c* release and apoptosis in myenteric neurons after oxaliplatin treatment [9]. Co-treatment with resveratrol significantly alleviated the translocation of nitrotyrosine when compared to the oxaliplatin-only treated group. Resveratrol has previously demonstrated inhibitory effects on peroxynitrite-mediated oxidation of proteins and lipids [43] and in prevention of nitrate and oxidative damage through upregulation of anti-oxidant enzyme superoxide dismutase isoforms [44] and activation of the Sirt1/AMPK and the Nrf2/anti-oxidant defence pathways [45].

4.2. Resveratrol ameliorates oxaliplatin-induced changes to distal colon morphology

Oxaliplatin administration was associated with a significant reduction in colonic smooth muscle thickness, which is similar to our previously published data [9]. As well as the effects on smooth muscle, oxaliplatin caused severe damage to colon morphology, one of these being abnormal vacuolisation within the intestinal crypts which has previously been reported to be an indicator of inflammation [46]. Co-administration of resveratrol with oxaliplatin had a protective effect on the colon, with the resveratrol + oxaliplatin-treated mice demonstrating healthy mucosal lining, crypt structure and muscle thickness. Resveratrol was previously reported to prevent GI inflammation [47, 48], but this is the first study to demonstrate protection from oxaliplatin-induced inflammation.

4.3. Resveratrol alleviates oxaliplatin-induced colonic dysfunction and constipation

Significant reduction in the amplitude of colonic contractions was observed in the colons from oxaliplatin-treated mice. This was associated with an increase in the number of pellets present in the colons of oxaliplatin-treated mice, indicating constipation due to insufficient propulsion of faecal pellets through the distal colon. These results, combined with

the increase in translocation of nitrated proteins to neuronal nuclei and previously reported loss of myenteric neurons [8, 9] as well as morphological changes in the colonic smooth muscles observed in the colons from oxaliplatin-treated mice, suggest that the decrease in the amplitude of colonic contractions is due to a decrease in neuronal input from motor neurons and less muscle mass available to perform contraction. Our observations are in agreement with previous studies reporting oxaliplatin-induced neuronal loss and alternations in nNOS expression leading to colonic dysmotility [8] and cisplatin-induced inhibition of intestinal transit [7]. Co-treatment with resveratrol alleviated oxaliplatin-induced changes in colonic contractile activity and symptoms of chronic constipation.

5. Conclusion

The results of this study demonstrate that oxaliplatin treatment causes neurotoxicity through nitrate translocations in the nuclei of enteric neurons and damages colon morphology. This damage is linked to motor dysfunction due to inhibition of the amplitude of colonic contractions leading to constipation. Co-treatment with resveratrol prevented neuronal toxicity and alleviated damage to GI mucosa, crypts and muscle layer resulting in improved contractility and a decrease in constipation. Resveratrol poses great therapeutic potential to prevent and alleviate oxaliplatin-induced oxidative damage to improve patient quality of life. Future research is required to determine the effects of resveratrol on the efficacy of oxaliplatin in an *in vivo* model of colorectal cancer and on the specific mechanisms used to infer its anti-oxidant/neuroprotective and anti-inflammatory actions.

Conflict of interest

The authors have no conflict of interest to declare

Contributors

ED undertook the experimental research and wrote the article, VA, LS, KN supervised ED and analysed data, edited and reviewed the article.

Ethical approval

All experimental work in this study was approved by the Victoria University Animal Experimentation Ethics Committee, VIC Australia and performed in accordance with the guidelines of the Australian National Health and Medical Research Council.

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Figure Legends

Figure 1. Effect of oxaliplatin treatment on enteric neurons and colon morphology.

Antibody against 3-Nitrotyrosine (3-NT) was used to label nitrated proteins within the myenteric plexus (A'-A'''). The number of neurons per 2 mm² displaying translocation of nitrated proteins to the nuclei was higher in the oxaliplatin-treated group compared to vehicle-treated. Resveratrol + oxaliplatin-treated mice showed significantly less translocation of 3-NT when compared to oxaliplatin. (B) Statistical analysis of the number of neurons displaying translocation of nitrated proteins into the nucleus. Data presented as mean ± standard error of the mean (S.E.M). *** $P < 0.001$ compared to all other groups (n=5 mice/group). (C'-C''') Gross morphological changes in the colon following repeated *in vivo* oxaliplatin administration. Mucosal damage (a), abnormal vacuolisation (b), crypt length (c), and muscle thickness were affected in oxaliplatin-treated mice (C''), these changes were prevented by co-treatment with resveratrol (C'''). (D) Statistical analysis of the muscle layer thickness in the colon preparations from treated mice. Data presented as mean ± S.E.M. * $P < 0.05$ compared to oxaliplatin-treated group, *** $P < 0.001$ compared to vehicle and resveratrol-only treated groups (n=5 mice/group, 10 sections per preparation from each animal).

Figure 2. Effect of oxaliplatin on contractility of the distal colon.

(A) A vertical slice map displaying the change in diameter of distal colon segments during peristaltic motility is presented. vehicle-treated (black), and resveratrol + oxaliplatin-treated (green) mice display similar diameters during relaxation and contraction and represent normal motor patterns. Colons from oxaliplatin-treated mice (red) display a reduction in the amplitude of colonic contractions. (B) Statistical analysis of changes in diameter of the distal colon from treated mice. Data presented as mean ± standard error of the mean (S.E.M). *** $P < 0.001$ compared to all other groups (n=5 mice/group). (C) Statistical analysis of the number of pellets present in the mouse colon. High numbers of pellets indicate constipation. * $P < 0.05$ compared to vehicle-treated group, *** $P < 0.001$ compared to all other groups (n=5 mice/group).

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