Acute paraquat exposure impairs colonic motility by selectively attenuating nitrergic signaling in the mouse.

Lucy Diss, Sarah Dyball, Tina Ghela, Jonathan Golding, Rachel Morris, Stephen Robinson, Rosemary Tucker, Talia Walter, Paul Young, Marcus Allen, Sara Fidalgo, Paul Gard, Jon Mabley, Bhavik Patel, Prabal Chatterjee and *Mark Yeoman.

School of Pharmacy and Biomolecular Sciences, Huxley Building, College of Life, Health and Physical Sciences, University of Brighton, Brighton, East Sussex, UK, BN2 4GJ.

#: These authors contributed equally to the study

Running Title: Paraquat impairs colonic motility in the mouse.

***Corresponding Author:**

Dr Mark S Yeoman

School of Pharmacy and Biomolecular Sciences

Huxley Building

University of Brighton

Brighton, East Sussex.

BN2 4GJ

Tel: +44 (0)1273 642078

E-mail: m.s.yeoman@brighton.ac.uk.

Abstract

Paraquat, a common herbicide, is responsible for large numbers of deaths worldwide through both deliberate and accidental ingestion. Previous studies have eluded that the bioavailability of paraquat increases substantially with increasing dose and that these changes may in part be due to the effects these high concentrations have on the gastrointestinal tract (GI tract). To date, the actions of acute, high concentrations (20 mM for 60 minutes) of paraquat on the GI tract, particularly the colon a major site of paraquat absorption, are unknown. This study examined the effects of acute paraquat administration on colonic motility in the C57BL/6 mouse. Acute paraquat exposure decreased colonic motility and the amplitude of colonic migrating motor complexes (CMMCs), major motor patterns involved in faecal pellet propulsion. In isolated segments of distal colon, paraquat increased resting tension and markedly attenuated electrical field stimulation-evoked relaxations. Pharmacological dissection of paraquat's mechanism of action on both the CMMCs and field stimulated tissue using the nitric oxide synthase inhibitor NG-nitro-L-Arginine and direct measurement of NO release from the myenteric plexus, demonstrated that paraquat selectively attenuates nitrergic signaling pathways. These changes did not appear to be due to alterations in colonic oxidative stress, inflammation or complex 1 activity, but were most likely caused by paraquat's ability to act as a redox couple. In summary, these data demonstrate that acute paraquat exposure attenuates colonic transit. These changes may facilitate the absorption of paraquat into the circulation and so facilitate its toxicity.

Key words: Colon motility, Colonic Migrating Motor Complex, Paraquat and Nitric Oxide.

1. Introduction

Paraquat is a widely used herbicide responsible for large numbers of deaths worldwide. Paraquat bioavailability is normally low (Chui et al., 1988; Gawarammana et al., 2011; Kan et al., 2010) but increases substantially with increasing doses, potentially due to effects on the colon a major site of paraquat absorption (Gawarammana et al., 2011). As reduced motility may provide a mechanism to facilitate paraquat absorption and increase its bioavailability, we have chosen to examine the effects of paraquat on colonic motility.

Motility involves coordinated muscle contractions/relaxations. Contraction is driven by activity in excitatory motor neurons that release acetylcholine and tachykinins (Gamage et al., 2013; Wade et al., 2004). Relaxation involves the activation of inhibitory motor neurons which release a combination of nitric oxide a purine (ATP or β-NAD) and vasoactive intestinal peptide (Akbulut et al., 2015; Cowen, 2000; Patel et al., 2014; Thrasivoulou et al., 2006). Changes in the balance between contraction and relaxation can lead to an impairment of motility.

Paraquat can alter cellular and organ function in a variety of ways. It is a pro-oxidant that generates the superoxide free radical (Day et al., 1999) and has been used to induce oxidative stress in a wide range of tissues/cell types to mimic disease (Bove et al., 2012; Djukic et al., 2012; Drechsel et al., 2008; McCormack et al., 2005; Miller et al., 2009; Samai et al., 2008) and to examine the role of oxidative stress in the natural ageing process (Jung et al., 2009; Salmon et al., 2009; Van Raamsdonk et al., 2009). Paraquat also reduces the activity of protein complexes of the respiratory chain

(Cocheme et al., 2008; Gomez et al., 2007; Rodriguez-Rocha et al., 2013) and can induce inflammation (Aires et al., 2013; Ajjuri et al., 2013; Bove et al., 2012).

This study examined the effect of paraquat on colonic motility and its mechanism of action.

2. Materials and Methods

2.1 Animals

All procedures were carried out according to U.K. Animals (Scientific Act), 1986 and associated guidelines and were approved by the University of Brighton Ethics Committee. Male C57BL/6J mice were obtained from Harlan UK at 8 weeks of age and housed in groups of 3-4 until required. Animals were maintained at 19.0 ± 1 °C, 55 % humidity and fed on a maintenance diet (RM1 (E) 801002 chow, Special Diet Services) and had free access to water. The animals were kept on a 12 hour light/dark cycle and studied at 3-4 months of age. Mice were killed prior to experimentation by CO_2 (100%) asphyxiation, followed by cervical dislocation.

2.2 Pellet motility assays

The whole colon was harvested and placed in ice cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer solution, pH 7.4 containing (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose). The mesentery was trimmed using fine scissors and the whole colon was then loosely pinned in a Sylgard-lined flow bath, allowing a lateral movement of approximately 0.5 cm about the mid-line and perfused with oxygenated Krebs buffer solution at 37 ± 1 °C at a flow rate of 8 ml min^{-1}. A small (2 mm) incision was made in both ends of the colon and the openings pinned flat to facilitate pellet insertion and its expulsion at the distal end. If spontaneous evacuation was not achieved, the faecal pellets were removed from the isolated colon after 30 minutes, by gently flushing the lumen of the colon with warmed Krebs buffer solution. The colon was then left to stabilize for 15 minutes, prior

to recordings of pellet motility. Measurements of motility were carried out using a 2mm diameter epoxy-coated artificial faecal pellet. The artificial faecal pellet was inserted 3-4 mm into the proximal end of the bowel using a fire-polished glass capillary and the movement of the pellet was monitored using a video camera. Pellet motility was tracked using Ethovision tracking software. Following a successful trial, the experiment was repeated two further times and the average response utilized for statistical analysis. Tissues were then perfused with 20 mM paraquat for 60 minutes and pellet motility assayed for a further three trials. The maximum time that any single trial was conducted was 45 minutes. The total transit time of the artificial faecal pellet was recorded along with the distance and the average velocity of the pellet determined (Patel et al., 2014).

2.3 Measurement of Colonic Migrating Motor Complexes (CMMCs)

Briefly the whole colon was placed in a Sylgard-lined recording chamber and a thin metal rod (1 mm diameter) placed through the lumen and secured at each end to the Sylgard. Recordings of circular muscle contractions were made at two locations along the whole isolated colon, one at the proximal end and one at the distal end. Fine suture silk was tied through the muscle layers at each location and connected to two separate isometric force transducers. The muscle was placed initially under a low level of tension 4 mN and then tension increased over the next 40 minutes until a final tension of 6 mN was reached. The signal from each force transducer then passed to a preamplifier and ADI Powerlab before being stored on computer using Chart software. The tissue was perfused for 60 minutes prior to recording with either normal Krebs buffer solution or Krebs buffer solution containing 20mM paraquat. Post this period, recordings of

spontaneous CMMCs were made for 60 minutes before the addition of 100 µM NG-nitro-L-Arginine (nitric oxide synthase inhibitor). The bath was allowed to equilibrate for 30 minutes with the NG-nitro-L-Arginine before spontaneous CMMCs were again recorded for 60 minutes.

2.4 Electrical field stimulated distal colon segments

The whole colon was removed and 2 cm sections of distal colon were hung vertically in an organ bath containing Krebs buffer solution. Distal colon segments were chosen as their pharmacology in mice is far better understood than the proximal colon. Tissues were then incubated for 60 minutes in either control Krebs buffer or Krebs buffer containing 20mM paraquat. At the end of this period the tissue was washed 4 times and 10 µM guanethidine added to the bath. The tissue was then dosed up with 100 µM acetylcholine for 1 minute every 10 minutes, until successive applications yielded a consistent response. Following this a frequency response curve was generated by passing current pulses across the tissue (40 V, 0.3 ms pulse duration, 0.1-30 Hz). Tissues were stimulated for 30 s every 5 minutes. The tissues were then washed and the frequency response curves repeated in the presence of either 100 µM NG-nitro-L-Arginine or 1 µM scopolamine (muscarinic antagonist) + 1µM GR159897 (NK_2 receptor antagonist) to block contractile pathways.

Responses to EFS can be obtained through activation of neurons within the plexi or directly through activation of the smooth muscle. To ensure our protocol was selectively activating neurons 400 nM tetrodotoxin was added to the tissue to block neuronal voltage –gated Na^+ channels and the tissue again stimulated at 10 Hz.

137 *2.5 Detection of NO release from the myenteric plexus*

138 Methods for the detection of NO release from the myenteric plexus have been
139 described previously (MacEachern et al., 2011; Patel et al., 2010). 10µM veratridine
140 (Na^+ channel activator) was used to evoke NO release to mimic the effects of electrical
141 field stimulation.

142 For the following assays freshly isolated distal colonic segments were placed in either
143 Krebs buffer solution or Krebs buffer solution containing 20 mM paraquat for 60
144 minutes. Following the incubation the tissues were washed and the colon bisected
145 along the mesenteric border to expose the mucosal tissue, which was then scraped
146 away. The mucosa and remaining muscle layers were then stored separately.

147 *2.6 Malondialdehyde assay*

148 Malondialdehyde formation was utilized to quantify levels of lipid peroxidation in the
149 tissue samples and measured as thiobarbituric acid-reactive material. Tissues were
150 homogenized (100 mg ml^{-1}) in 1.15% KCl buffer. 200 µl of the homogenates were then
151 added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 µl 8.1%
152 sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5) and 600µl distilled H_2O. The
153 mixture was then heated at 90 °C for 45 minutes. After cooling to room temperature,
154 the samples were cleared by centrifugation (10000g, 10 minutes) and their absorbance
155 measured at 532 nm, using 1, 1, 3, 3-tetramethoxypropane as an external standard.
156 The level of lipid peroxides was expressed as nmol MDA /mg protein (Bradford assay).

157 *2.7 Western Blot*

158 The mucosa and remaining muscle layers were separately snap frozen in liquid N_2 for
159 storage. Tissue was placed on ice in lysis buffer (10 mM HEPES, 150 mM NaCl, 1mM
160 EDTA, 0.2 % Nonidet P40, protease inhibitor cocktail P8340, Sigma-Aldrich Inc.) and
161 manually lysed. Lysates were centrifuged for 10 minutes, at 4 °C at 700 x g to pellet
162 debris and nuclear components. Supernatants were removed and their protein content
163 assessed using Quick Start Bradford Dye reagent (Bio-Rad) 26. 15 µg of protein from
164 each sample was combined with an equal volume of 2 x Laemmli loading buffer (S3401,
165 Sigma Aldrich), separated on a 10 % SDS-PAGE gel using the Mini-protean II
166 electrophoresis cell (Bio-Rad) and transferred to Immobilon polyvinylidene fluoride
167 (PVDF) membrane using the Trans-Blot® wet transfer (Bio-Rad). Membranes were
168 blocked with 5 % milk in PBS-Tween 20 (0.2 %) for 3 hours and then incubated
169 overnight at 4 °C with either a rabbit anti-TNFα antibody (1:5000; Millipore) or a rabbit
170 anti-complex 1 antibody (1:5000; Aviva systems) or a mouse monoclonal anti-actin
171 antibody (1:5000; Santa Cruz) diluted in milk-PBS-Tween 20. Membranes were
172 washed five times in PBS-Tween 20 and then incubated with goat anti-rabbit HRP-
173 conjugated secondary antibody at 1:2000 (SC-2005, Santa Cruz Biotechnology) or a
174 goat anti-mouse HRP-conjugated secondary (1:2500) for 1 hr at room temperature.
175 After five further washes with PBS-Tween 20, membranes were treated with
176 Amersham™ ECL plus western blotting detection system (GE Healthcare) and exposed
177 to Amersham™ ECL plus film. The relative intensity of each SERT band was measured
178 by densitometry using the Fluorochem™ imager (Alpha Innotech) and the signal was
179 normalized to the density of the corresponding actin labelled band.

180 *2.8 Complex 1 assay*

A complex I enzymatic activity microplate assay kit (Mitoscience, Eugene, Oregon) was used to determine the activity of the complex I. Briefly, mitochondrial OXPHOS Complex I was immunocaptured and the activity was determined at 450 nm by following the oxidation of NADH to NAD^+. Total protein in the mitochondrial fraction was estimated using a protein assay kit (Bradford).

2.9 Data Analysis

Pellet velocities were calculated by calculating the distance moved in 500 s. CMMC parameters were calculated as follows. Amplitude was determined by measuring the change in tension from the trough to peak of the CMMC. Duration represented the width of the CMMC at baseline in seconds. Velocity was determined by calculating the time from the peak of the CMMC in the proximal colon to the peak of the CMMC in the distal colon and dividing it by the distance between the two transducers. Values for each of these three parameters were obtained for at least 6 CMMCs and averaged to give a mean value for each tissue. CMMC frequency was determined by calculating the time taken for 6 spontaneous CMMCs and dividing by 6.

In experiments that examined the effects of paraquat on electrical field stimulation evoked colonic responses, alterations in resting tension were determined during the initial 60 minute stabilization period when the tissues were either bathed in Krebs buffer solution or Krebs buffer solution containing 20 mM paraquat. Tension changes were determined by measuring the difference in baseline tension at time zero from that at time 60 minutes. The number of contractions per 10 minutes was determined by

counting the number of phasic contractions that occurred during the final 10 minutes of the 60 minute stabilization period. Results from the electrical field stimulation tissue experiments are plotted as the integral of the response calculated during the 30 second stimulation period using GraphPad Prism and averaging the responses from the 5 preparations. Statistical analysis of the pellet motility, resting tension and number of contractions per minute, the analysis of malondialdehyde levels and the Western blots were compared using an unpaired t-test. Changes in NO release were compared using a paired t-test. CMMC responses were compared using a 1 way ANOVA with a post-hoc Tukey test. Electrical field stimulation -evoked responses were analyzed using a two-way ANOVA with frequency and paraquat as the two variables. Post hoc analysis was carried out using a Tukey test. All data are expressed as the mean±SEM and $p<0.05$ was taken as significant.

3. Results

Isolated full thickness segments of distal colon maintained in Krebs buffer slowly relaxed over a 60 minute period to a stable resting tension (Fig 1Ai). In the presence of paraquat tissues showed a significant increase in both the resting tension (Fig 1Aii/B) and the number of large amplitude spontaneous contractions per 10 minutes (Fig 1Aii/C). Field stimulation evoked a relaxation of untreated tissue at frequencies between 1-10 Hz (Fig 2Ai/Aiii). However, a marked contraction was observed in paraquat-treated tissue stimulated over the same frequency range (Fig. 2 Aii/Aiii; $p<0.001$, 2-way ANOVA). Application of L-NNA to block nitrergic signaling the main component of the relaxation response led to a contractile response being recorded in both treated and untreated tissues that were not significantly different (Fig 2B). In a similar series of experiments blockade of the contractile component of the response with a mixture of scopolamine and GR159897, increased the amplitude of the relaxation seen in untreated tissue (Fig 2Ci/Ciii), but completely blocked the response of the paraquat-treated tissue to field stimulation (Fig 2Cii/Ciii; $p<0.001$, 2-way ANOVA). No electrical field stimulation-evoked responses were observed in the presence of TTx confirming our stimulation protocol was selectively activating neurons (data not shown). These data strongly suggested that paraquat was inhibiting electrical field stimulation - evoked nitrergic signaling without affecting the contractile component of the response.

In order to test whether paraquat treatment also affected motility we examined the effects of paraquat on CMMCs a major motor pattern involved in pellet motility. CMMCs are propagating waves of contraction that typically travel in an oral to anal direction down the colon. In control preparations 80% of recorded CMMCs from 6 preparations

241 migrated in an oral to anal direction with the remaining 20% being uncoordinated. In
242 paraquat-treated tissue only 50% of CMMCs moved in an oral to anal direction with 20
243 % moving in a retrograde direction and 30 % being uncoordinated. The changes
244 observed with paraquat were similar to those seen with L-NNA where 40% moving in an
245 oral to anal direction, with 20% moving in a retrograde direction and 40% being
246 uncoordinated. Pre-treatment with paraquat caused a significant decrease in the
247 amplitude of spontaneous CMMCs in both the proximal and distal colons ($p<0.05$; Fig
248 3A/Bi/Ci). Paraquat reduced the duration of CMMC in the distal colon ($p<0.05$) but was
249 without effect on the duration of the proximal colon (Fig 3Bii/Cii). The frequency of
250 CMMCs was increased following paraquat application ($p<0.05$; Fig 3D), although there
251 was no change in the velocity (Fig. 3E). Subsequent application of L-NNA following
252 paraquat treatment failed to evoke any additional changes. Based on the EFS data
253 shown above, one possible explanation for these data was that paraquat was blocking
254 the L-NNA sensitive nitrergic component of the colonic migrating motor complexes. To
255 test this hypothesis, the effects of L-NNA administration on the properties of CMMCs
256 were examined in a separate series of experiments. L-NNA decreased CMMC
257 amplitude (Fig 4A; $p<0.05$), distal duration (Fig 4B; $p<0.05$) and increased CMMC
258 frequency (Fig 4C; $p<0.05$), without affecting velocity (Fig 4D), changes that were
259 consistent with paraquat inhibiting nitrergic signaling.

260 We next examined whether paraquat altered the migration velocity of an artificial faecal
261 pellet placed in *ex vivo* full length colons. All colons examined completely evacuated
262 their natural pellets within 30 minutes (n=4). Under control conditions the 2 mm artificial
263 pellet moved in a stepwise manner along the colon (Fig 5Ai; black lines), with a mean

velocity of 0.73 ± 0.23 cm min^{-1} (Fig 5Aii). Addition of paraquat inhibited stepwise movements of the pellet (Fig 5Ai; grey lines) and significantly reduced mean velocity to 0.004 ± 0.008 cm min^{-1} ($p<0.01$; Fig 5Aii).

To examine whether paraquat inhibited NO production, amperometric measurements were made from the myenteric plexus following application of veratradine a voltage-gated Na^+ channel agonist. Application of paraquat was observed to cause a significant decrease in the amperometric current (Fig 6A/B).

In order to examine the mechanism by which acute paraquat treatment reduced colonic motility we examined whether the observed effects were linked to changes in oxidative stress, complex 1 expression/activity or the production of TNFalpha a pro-inflammatory mediator. These putative mechanisms of action were chosen as chronic paraquat has previously been shown to evoke these changes in other systems. These experiments were carried out solely on the distal colon segments to ensure consistency with the EFS data. A determination of MDA levels, a marker of lipid peroxidation, in both the mucosa and muscle of the distal colon showed that paraquat pretreatment increased levels of MDA in the mucosa but was without significant effect in the muscle (Fig 1 suppl.), strongly suggesting that oxidative stress was not responsible for the observed changes in muscle function.

Analysis of levels of the cytokine, TNFα, a marker of cellular inflammation demonstrated a non-significant decrease in levels in both the mucosa and muscle samples (Fig 2A suppl.), suggesting that the observed changes in function were not caused by increases in inflammation. Finally the expression and activity of Complex 1 was examined.

Western blot analysis showed no significant changes in the expression of Complex 1 in both the mucosa and muscle samples from the distal colon (Fig 2B suppl). Measurements of complex 1 activity also failed to show a significant change following paraquat pre-treatment (data not shown).

4. Discussion

The current study has shown that acute paraquat treatment can reduce colonic motility in the mouse. To our knowledge this is the first report of paraquat affecting GI tract motility. The colon has previously been shown to be a major route by which paraquat is absorbed into the blood stream and a reduction in lower bowel motility would increase the time that paraquat spends in the lower bowel facilitating its absorption, its bioavailability and the potential for toxicity.

4.1 Paraquat selectively inhibits nitrergic signaling pathways in colonic smooth muscle.

Several lines of evidence strongly infer that these changes are due to paraquat inhibiting NO signaling in the colon. First, in isolated segments of full thickness colon application of paraquat was capable of increasing the resting tension of the muscle and inhibiting field stimulated relaxations, effects that were mimicked by pretreatment with L-NNA (Dickson et al., 2010). Second, pretreatment with paraquat increased the frequency and decreased the amplitude of the CMMCs, changes that could be mimicked by the application of L-NNA a NOS inhibitor. In support of this observation, a previous study has shown similar changes in the properties of the CMMCs in wild type mice following application of L-NNA (Duncan et al., 2013; Mawe et al., 2006). Third, we have shown that acute paraquat treatment can inhibit pellet motility. Although we have not demonstrated that this is due directly to paraquat inhibiting nitrergic signaling, the results are consistent with previous studies that have demonstrated that application of L-NNA was able to reduce pellet motility in an intact but isolated colon consistent with the results of the current study (Fida et al., 1997; Mawe et al., 2006). Similarly, in NOS^-

$^{/-}$ mice, pellet motility has also been shown to be inhibited (Coates et al., 2004; Mawe et al., 2006). These changes were shown to be due to L-NNA removing a tonic inhibition from the muscle allowing the muscle cells to depolarize which in turn caused CMMCs to become uncoordinated or to travel in a retrograde direction. Paraquat caused similar changes to CMMC migration in the current study and these, together with a marked decrease in CMMC amplitude were most likely responsible for the decrease in pellet motility despite a lack of change in CMMC velocity.

To identify the site of action of paraquat we used amperometric measurements and demonstrated that paraquat almost completely inhibited NO release from the myenteric plexus. Paraquat has been shown to cause muscle relaxation through the activation of soluble guanylate cyclase and activation of SK channels. It is possible that paraquat could also be interfering with postsynaptic signal transduction pathways; however, the almost complete inhibition of NO release suggests that if this is the case then it is a minor component of paraquats' effect on the colon.

4.2 How does paraquat attenuate NO release?

Previous studies have shown that paraquat is capable of inducing oxidative stress in tissues and this has been shown to be the main way in which it exerts its toxicity in humans (Dinis-Oliveira et al., 2008; Drechsel et al., 2008). However, it is difficult to see how the observed selectivity for nitrergic signaling can be explained by this mechanism. Analysis of MDA levels, a marker of lipid peroxidation, failed to show any significant changes in the colonic smooth muscle, although significant increases were observed in the mucosa. Previous studies have demonstrated that signaling via the mucosa is an

important regulator of pellet motility and CMMCs, providing the possibility that the observed increases in oxidative stress could affect pellet motility (Bischoff et al., 2009; Ghia et al., 2009). However, mucosal signaling pathways affect both the contractile and relaxation pathways and therefore the increase in mucosal oxidative stress is unlikely to provide an explanation for the selective attenuation of nitrergic signaling pathways (Idzko et al., 2004; Margolis et al., 2014). Based on these data it is unlikely that oxidative stress is the main cause of the changes observed in this study. Paraquat can also induce inflammation through the production of a range of cytokines including TNFalpha and these changes could contribute to the attenuation in nitrergic signaling (Aires et al., 2013; Amirshahrokhi, 2013). Expression of the pro-inflammatory cytokine, TNFalpha was reduced non-significantly in the both the mucosa and muscle, inconsistent with inflammation driving the observed changes in signaling. Finally, paraquat has previously been shown to interfere with the activity of Complex 1 a respiratory chain enzyme (Choi et al., 2008; Cocheme et al., 2008; Rodriguez-Rocha et al., 2013). However, pretreatment with paraquat failed to alter both the expression and activity of the enzyme negating this as a possible mechanism of action.

The lack of any significant changes in these pathways is probably indicative of the relatively short period that the tissue was exposed to paraquat (60 minutes). In the light of these findings a single previous study by Day et al has inferred an alternative mechanism that may help explain how paraquat selectively suppresses NO signaling pathways in the colon (Day et al., 1999). Nitric oxide selectively oxidises L-arginine to yield L-citrulline and the free radical gas NO. An additional byproduct of this reaction is the superoxide anion. The authors showed that in the presence of paraquat the NOS

enzyme was capable of generating a paraquat free radical demonstrating that NOS shunts electrons to paraquat, thereby uncoupling the enzyme and inhibiting NO production (Day et al., 1999; Moran et al., 2010). The reduction in NO production was shown to be associated with an increased production of the superoxide anion and the consequential production of hydrogen peroxide. This provides a mechanism by which paraquat could increase oxidative stress. Our lack of an observed change in the oxidative stress marker MDA could be due to the limited time period that tissue was exposed to paraquat or could be due to the fact that the changes were initially limited to a subset of neurons in the colon causing paraquats' effect to be diluted out by a lack of lipid peroxidation in the bulk tissue.

4.3 Conclusions

We have demonstrated that acute pre-treatment with high doses of paraquat is capable of inhibiting colonic transit and that these changes do not appear to reflect appropriate alterations in oxidative damage, inflammation or Complex 1 activity. These short-term changes most likely reflect the ability of paraquat to inhibit the production of NO by acting as a redox couple. These data are important as they suggest that agents designed to relax GI smooth muscle such as VIP or the purine ATP or β-NAD may be useful targets to help maintain normal colonic movements in conditions of paraquat poisoning. Additionally, many studies in cell culture routinely use short duration administration of high concentrations of paraquat as a means of inducing oxidative stress and cell damage in an attempt to either mimic disease or the ageing process. It is clear that caution needs to be taken when interpreting these results as it is possible that any short-term changes that are observed maybe happening in the absence of

oxidative stress or at least may be a combination of oxidative stress and an inhibition of nitrergic signaling.

Acknowledgements

We would like to thank the University of Brighton for funding this project and the staff of the Bioresource Centre for their help in this study.

References

Aires, R.D., Capettini, L.S.A., Silva, J.F., Rodrigues-Machado, M.d.G., Pinho, V., Teixeira, M.M., Cortes, S.F., Lemos, V.S. 2013. Paraquat Poisoning Induces TNF-alpha-Dependent iNOS/NO Mediated Hyporesponsiveness of the Aorta to Vasoconstrictors in Rats. Plos One 8.

Ajjuri, R.R., O'Donnell, J.M. 2013. Novel whole-tissue quantitative assay of nitric oxide levels in Drosophila neuroinflammatory response. Journal of visualized experiments : JoVE, 50892-50892.

Akbulut, K., Aktas, S., Akbulut, H. 2015. The role of melatonin, sirtuin2 and FoXO1 transcription factor in the aging process of colon in male rats. Biogerontology 16, 99-108.

Amirshahrokhi, K. 2013. Anti-inflammatory effect of thalidomide in paraquat-induced pulmonary injury in mice. International Immunopharmacology 17, 210-215.

Bischoff, S.C., Mailer, R., Pabst, O., Weier, G., Sedlik, W., Li, Z., Chen, J.J., Murphy, D.L., Gershon, M.D. 2009. Role of serotonin in intestinal inflammation: knockout of serotonin reuptake transporter exacerbates 2,4,6-trinitrobenzene sulfonic acid colitis in mice, G685-G695.

Bove, J., Perier, C. 2012. Neurotoxin based models of Parkinson's disease. Neuroscience 211, 51-76.

Choi, W.-S., Kruse, S.E., Palmiter, R.D., Xia, Z. 2008. Mitochondrial complex I inhibition is not required for dopaminergic neuron death induced by rotenone, MPP+, or paraquat. Proceedings of the National Academy of Sciences of the United States of America 105, 15136-15141.

Chui, Y.C., Poon, G., Law, F. 1988. Toxicokinetics and bioavailability of paraquat in rats following different routes of administration. Toxicology and Industrial Health 4, 203-219.

Coates, M.D., Mahoney, C.R., Linden, D.R., Sampson, J.E., Chen, J., Blaszyk, H., Crowell, M.D., Sharkey, K.A., Gershon, M.D., Mawe, G.M., Moses, P.L. 2004. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. Gastroenterology 126, 1657-1664.

Cocheme, H.M., Murphy, M.P. 2008. Complex I is the major site of mitochondrial superoxide production by paraquat. Journal of Biological Chemistry 283, 1786-1798.

Cowen, T. 2000. Symposium on Mechanisms of Ageing and Longevity. Journal of Anatomy 197, i-i.

Day, B.J., Patel, M., Calavetta, L., Chang, L.Y., Stamler, J.S. 1999. A mechanism of paraquat toxicity involving nitric oxide synthase. Proceedings of the National Academy of Sciences of the United States of America 96, 12760-12765.

Dickson, E.J., Heredia, D.J., McCann, C.J., Hennig, G.W., Smith, T.K. 2010. The mechanisms underlying the generation of the colonic migrating motor complex in both wild-type and nNOS knockout mice. American Journal of Physiology-Gastrointestinal and Liver Physiology 298, G222-G232.

Dinis-Oliveira, R.J., Duarte, J.A., Sanchez-Navarro, A., Remiao, F., Bastos, M.L., Carvalho, F. 2008. Paraquat poisonings: Mechanisms of lung toxicity, clinical features, and treatment. Critical Reviews in Toxicology 38, 13-71.

Djukic, M.M., Jovanovic, M.D., Ninkovic, M., Stevanovic, I., Ilic, K., Curcic, M., Vekic, J. 2012. Protective role of glutathione reductase in paraquat induced neurotoxicity. Chemico-Biological Interactions 199, 74-86.

Drechsel, D.A., Patel, M. 2008. Role of reactive oxygen species in the neurotoxicity of environmental agents implicated in Parkinson's disease. Free Radical Biology and Medicine 44, 1873-1886.

Duncan, S.H., Flint, H.J. 2013. Probiotics and prebiotics and health in ageing populations. Maturitas 75, 44-50.

Fida, R., Lyster, D.J.K., Bywater, R.A.R., Taylor, G.S. 1997. Colonic migrating motor complexes (CMMCs) in the isolated mouse colon. Neurogastroenterology and Motility 9, 99-107.

Gamage, P.P.K.M., Ranson, R.N., Patel, B.A., Yeoman, M.S., Saffrey, M.J. 2013. Myenteric neuron numbers are maintained in aging mouse distal colon. Neurogastroenterology & Motility 25, e495-e505.

Gawarammana, I.B., Buckley, N.A. 2011. Medical management of paraquat ingestion. British Journal of Clinical Pharmacology 72, 745-757.

Ghia, J.E., Li, N., Wang, H., Collins, M., Deng, Y., El–Sharkawy, R.T., Côté, F., Mallet, J., Khan, W.I. 2009. Serotonin Has a Key Role in Pathogenesis of Experimental Colitis. Gastroenterology 137, 1649-1660.

Gomez, C., Bandez, M.J., Navarro, A. 2007. Pesticides and impairment of mitochondrial function in relation with the parkinsonian syndrome. Frontiers in Bioscience 12, 1079-1093.

Idzko, M., Panther, E., Stratz, C., Müller, T., Bayer, H., Zissel, G., Dürk, T., Sorichter, S., Di Virgilio, F., Geissler, M., Fiebich, B., Herouy, Y., Elsner, P., Norgauer, J., Ferrari, D. 2004. The

Serotoninergic Receptors of Human Dendritic Cells: Identification and Coupling to Cytokine Release. The Journal of Immunology 172, 6011-6019.

Jung, T., Hoehn, A., Catalgol, B., Grune, T. 2009. Age-related differences in oxidative protein-damage in young and senescent fibroblasts. Archives of Biochemistry and Biophysics 483, 127-135.

Kan, X., Zhang, X.-Y., Dong, J., Li, W.-S., Hu, G.-X., Lu, Z.-Q. 2010. Toxicokinetics of paraquat in rabbits. Chinese journal of industrial hygiene and occupational diseases 28, 756-759.

MacEachern, S.J., Patel, B.A., McKay, D.M., Sharkey, K.A. 2011. Nitric oxide regulation of colonic epithelial ion transport: a novel role for enteric glia in the myenteric plexus. The Journal of Physiology 589, 3333-3348.

Margolis, K.G., Stevanovic, K., Li, Z., Yang, Q.M., Oravecz, T., Zambrowicz, B., Jhaver, K.G., Diacou, A., Gershon, M.D. 2014. Pharmacological reduction of mucosal but not neuronal serotonin opposes inflammation in mouse intestine. Gut 63, 928-937.

Mawe, G.M., Coates, M.D., Moses, P.L. 2006. Review article: intestinal serotonin signalling in irritable bowel syndrome. Alimentary Pharmacology & Therapeutics 23, 1067-1076.

McCormack, A.L., Atienza, J.G., Johnston, L.C., Andersen, J.K., Vu, S., Di Monte, D.A. 2005. Role of oxidative stress in paraquat-induced dopaminergic cell degeneration. Journal of Neurochemistry 93, 1030-1037.

Miller, R.L., James-Kracke, M., Sun, G.Y., Sun, A.Y. 2009. Oxidative and Inflammatory Pathways in Parkinson's Disease. Neurochemical Research 34, 55-65.

Moran, J.M., Ortiz-Ortiz, M.A., Ruiz-Mesa, L.M., Fuentes, J.M. 2010. Nitric Oxide in Paraquat-Mediated Toxicity: A Review. Journal of Biochemical and Molecular Toxicology 24, 402-409.

Patel, B.A., Dai, X., Burda, J.E., Zhao, H., Swain, G.M., Galligan, J.J., Bian, X. 2010. Inhibitory neuromuscular transmission to ileal longitudinal muscle predominates in neonatal guinea pigs. Neurogastroenterology & Motility 22, 909-e237.

Patel, B.A., Patel, N., Fidalgo, S., Wang, C., Ranson, R.N., Saffrey, M.J., Yeoman, M.S. 2014. Impaired colonic motility and reduction in tachykinin signalling in the aged mouse. Experimental Gerontology 53, 24-30.

Rodriguez-Rocha, H., Garcia-Garcia, A., Pickett, C., Li, S., Jones, J., Chen, H., Webb, B., Choi, J., Zhou, Y., Zimmerman, M.C., Franco, R. 2013. Compartmentalized oxidative stress in dopaminergic cell death induced by pesticides and complex I inhibitors: Distinct roles of superoxide anion and superoxide dismutases. Free Radical Biology and Medicine 61, 370-383.

514 Salmon, A.B., Perez, V.I., Bokov, A., Jernigan, A., Kim, G., Zhao, H., Levine, R.L., Richardson, A.
515 2009. Lack of methionine sulfoxide reductase A in mice increases sensitivity to oxidative stress
516 but does not diminish life span. Faseb Journal 23, 3601-3608.
517
518 Samai, M., Hague, T., Naughton, D.P., Gard, P.R., Chatteriee, P.K. 2008. Reduction of paraquat-
519 induced renal cytotoxicity by manganese and copper complexes of EGTA and EHPG. Free
520 Radical Biology and Medicine 44, 711-721.
521
522 Thrasivoulou, C., Soubeyre, V., Ridha, H., Giuliani, D., Giaroni, C., Michael, G.J., Saffrey, M.J.,
523 Cowen, T. 2006. Reactive oxygen species, dietary restriction and neurotrophic factors in age-
524 related loss of myenteric neurons. Aging Cell 5, 247-257.
525
526 Van Raamsdonk, J.M., Hekimi, S. 2009. Deletion of the Mitochondrial Superoxide Dismutase
527 sod-2 Extends Lifespan in Caenorhabditis elegans. Plos Genetics 5.
528
529 Wade, P.R., Cowen, T. 2004. Neurodegeneration: a key factor in the ageing gut.
530 Neurogastroenterology & Motility 16, 19-23.

531

532

533

Figure Legends

Figure 1: Paraquat increases resting tension in murine distal colon. Sample traces of the force generated by isolated segments of distal colon maintained in Krebs buffer solution (Ai) or Krebs buffer solution containing 20mM paraquat (Aii). Bar graphs illustrating that paraquat increases resting tension (B) and the number of phasic contractions per minute (C) of distal colon segments. N=6 for each group; ***P<0.001.

Figure 2: Paraquat inhibits electrical field stimulated relaxations in murine distal colon. Sample traces of the force generated following a 30 s, 5 Hz stimulation of segments of distal colon maintained in Krebs buffer solution (Ai, Bi, Ci) or 20 mM paraquat (Aii, Bii, Cii) in the presence and absence of either L-NNA (Bi, Bii) or scopolamine and GR159897 (Ci, Cii). Frequency response curve illustrating that paraquat pre-treatment inhibits electrically evoked relaxation of the tissue (Aiii, Ciii) without affecting electrically evoked contractions (Biii). N=5 for each group, * p<0.05; ** p<0.01; *** p<0.001.

Figure 3: Effects of paraquat on the properties of colonic migrating motor complexes. Bar graphs illustrating the effects of paraquat on the amplitude (Ai, Bi); duration (Aii, Bii); frequency (C) and velocity (D) of CMMCs in isolated murine colon. N=6 for all groups; *p<0.05; **p<0.01; ***p<0.001 versus control.

Figure 4: Effects of paraquat on the properties of CMMCs are mimicked by inhibition of nitrergic signalling. Bar graphs illustrating the effects of L-NNA on the amplitude (Ai, Bi); duration (Aii, Bii); frequency (C) and velocity (D) of CMMCs in murine isolated colon. N=6 for all groups; *p<0.05; **p<0.01 versus control.

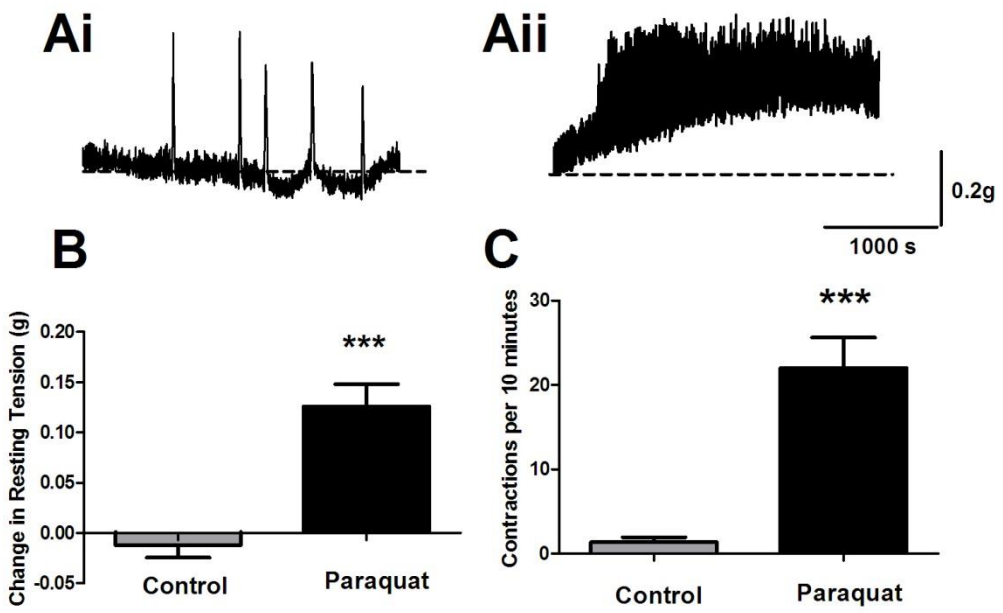
Figure 5: Paraquat inhibits faecal pellet motility. A) Traces illustrating typical distance time plots for the movement of an artificial faecal pellet through the intact but isolated colon. Control traces are shown in black, while paraquat pre-treated tissue is shown in grey. B) Bar graph showing the population data for the velocity of pellet movement through the colon. N=6 for each group; ** $p<0.01$ (Students T-test).

Figure 6: Paraquat decreases NO release from the myenteric plexus. A) Amperometric traces following application of 10µM veratridine to the distal colon myenteric plexus. B) NO release is impaired following paraquat treatment. N=4 for each group; ** $p<0.0001$.

564

565 **Fig. 1**

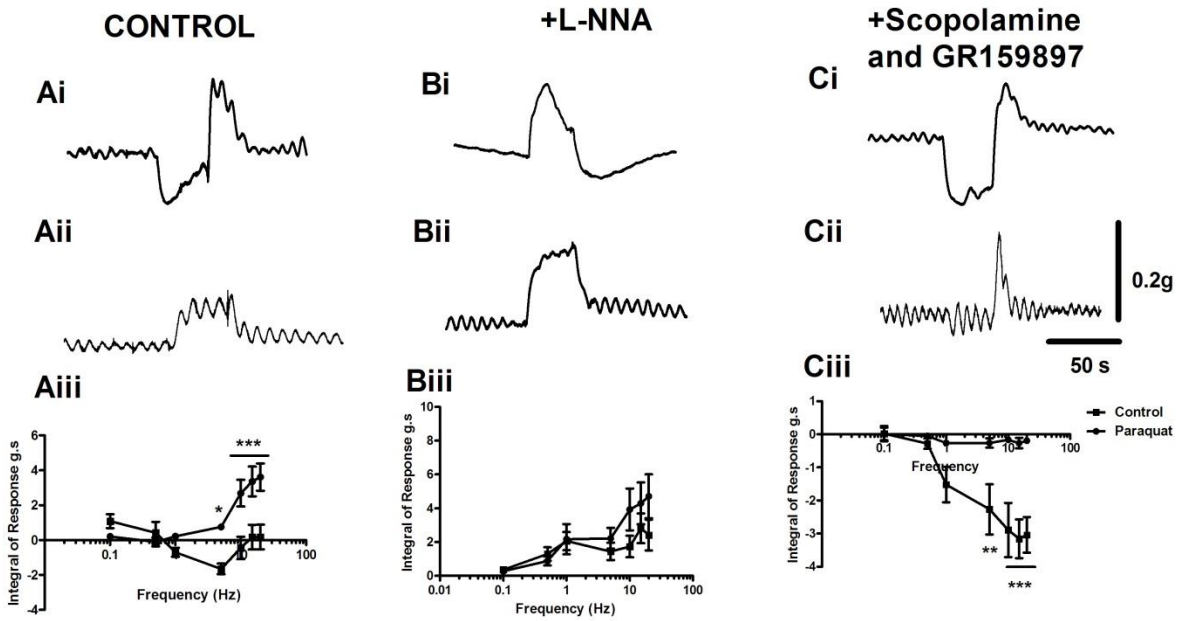
566



567

568

569 **Fig. 2**

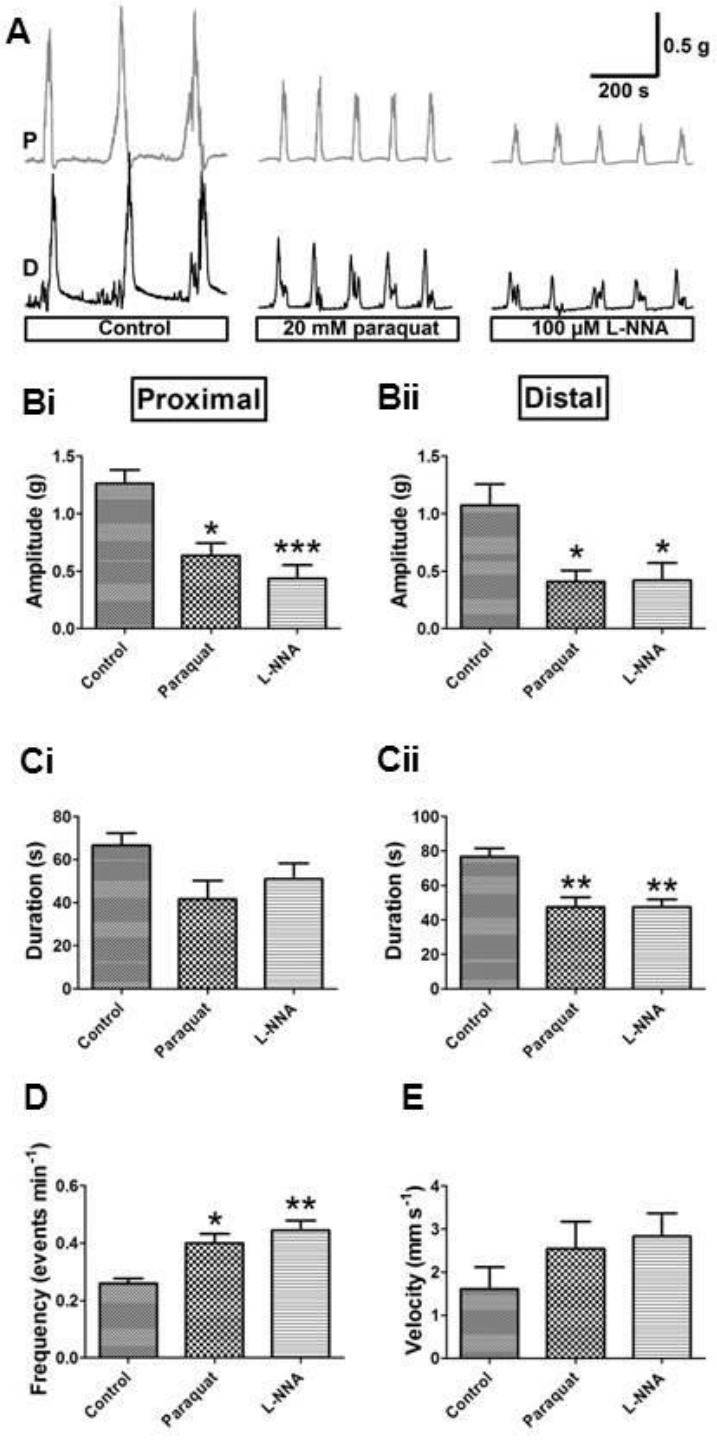


570

571

572 **Fig 3**

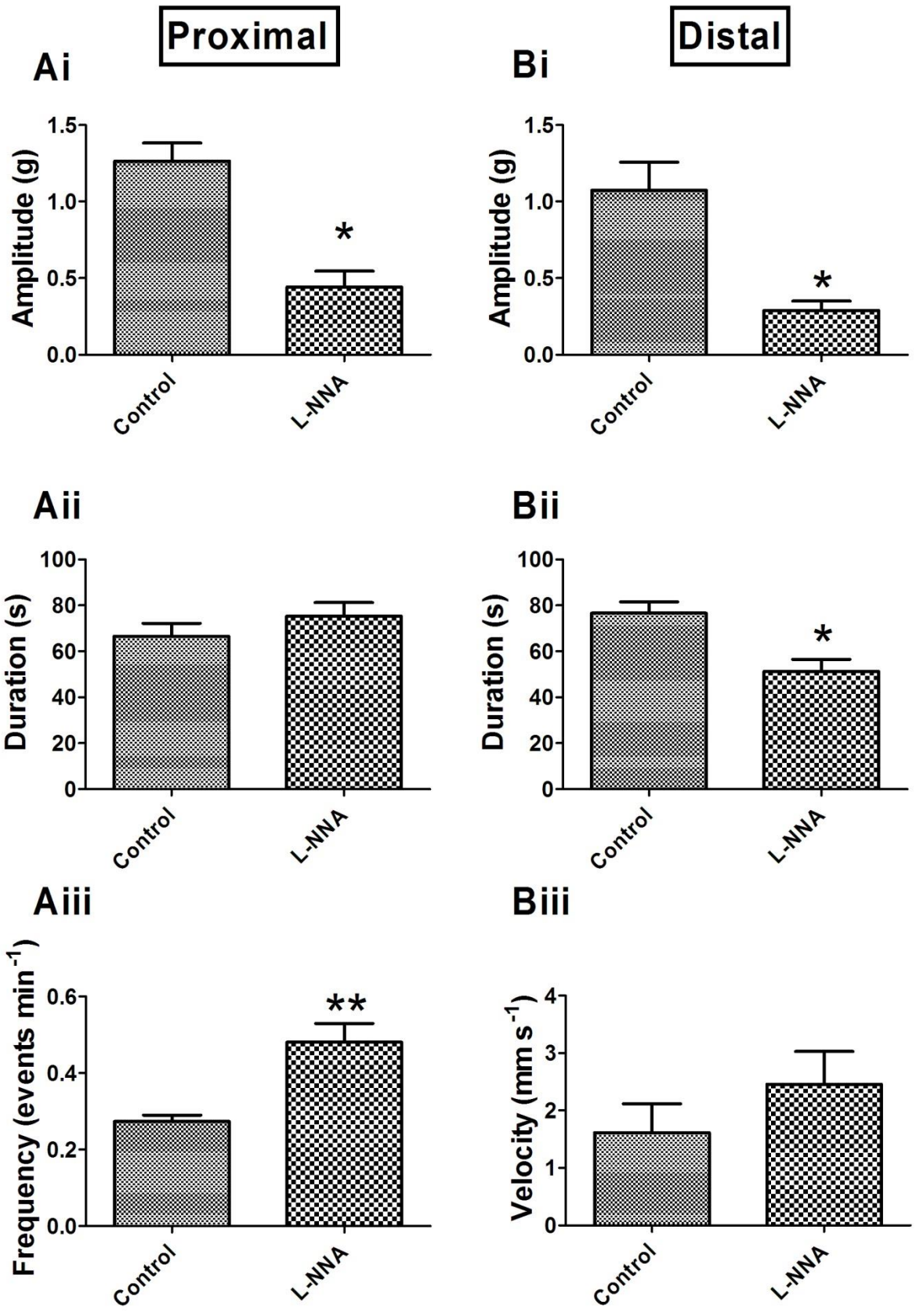
573


A
0.5 g
200 s
P
D
Control
20 mM paraquat
100 µM L-NNA
Bi
Proximal
Bii
Distal
Amplitude (g)
1.5
1.0
0.5
0.0
*
***
*
*
Control
Paraquat
L-NNA
Ci
Cii
Duration (s)
80
60
40
20
0
100
**
**
D
E
Frequency (events min-1)
0.6
0.4
0.2
0.0
*
**
Velocity (mm s-1)
4
3
2
1
0

574

575

576 **Fig 4**

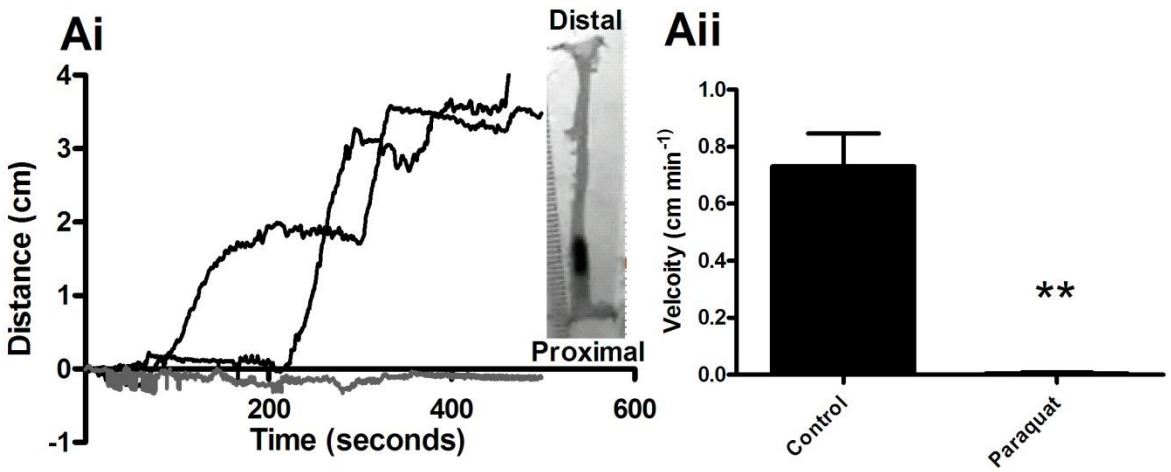

Proximal
Distal
Ai
Bi
Amplitude (g)
1.5
1.0
0.5
0.0
*
Control
L-NNA
Aii
Bii
Duration (s)
100
80
60
40
20
0
Aiii
Biii
Frequency (events min-1)
0.6
0.4
0.2
**
Velocity (mm s-1)
4
3
2
1

577

578

579 **Fig 5**

580

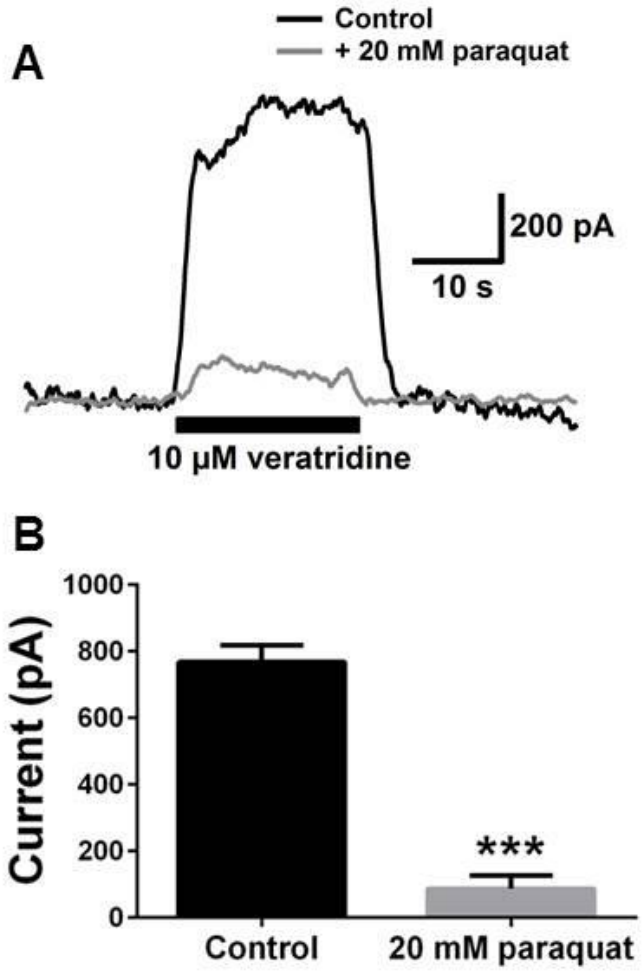


581

582

583 **Fig 6**

584


A
Control
+ 20 mM paraquat
200 pA
10 s
10 µM veratridine
B
Current (pA)
1000
800
600
400
200
0
***
Control
20 mM paraquat

585

586

Supplementary Information

Acute paraquat exposure impairs colonic motility by selectively attenuating nitrergic signaling in the mouse.

Lucy Diss, Sarah Dyball, Tina Ghela, Jonathan Golding, Rachel Morris, Stephen Robinson, Rosemary Tucker, Talia Walter, Paul Young, Marcus Allen, Sara Fidalgo, Paul Gard, Jon Mabley, Bhavik Patel, Prabal Chatterjee and *Mark Yeoman.

School of Pharmacy and Biomolecular Sciences, Huxley Building, College of Life, Health and Physical Sciences, University of Brighton, Brighton, East Sussex, UK, BN2 4GJ.

#: These authors contributed equally to the study

Running Title: Paraquat impairs colonic motility in the mouse.

***Corresponding Author:**

Dr Mark S Yeoman

School of Pharmacy and Biomolecular Sciences

Huxley Building

University of Brighton

Brighton, East Sussex.

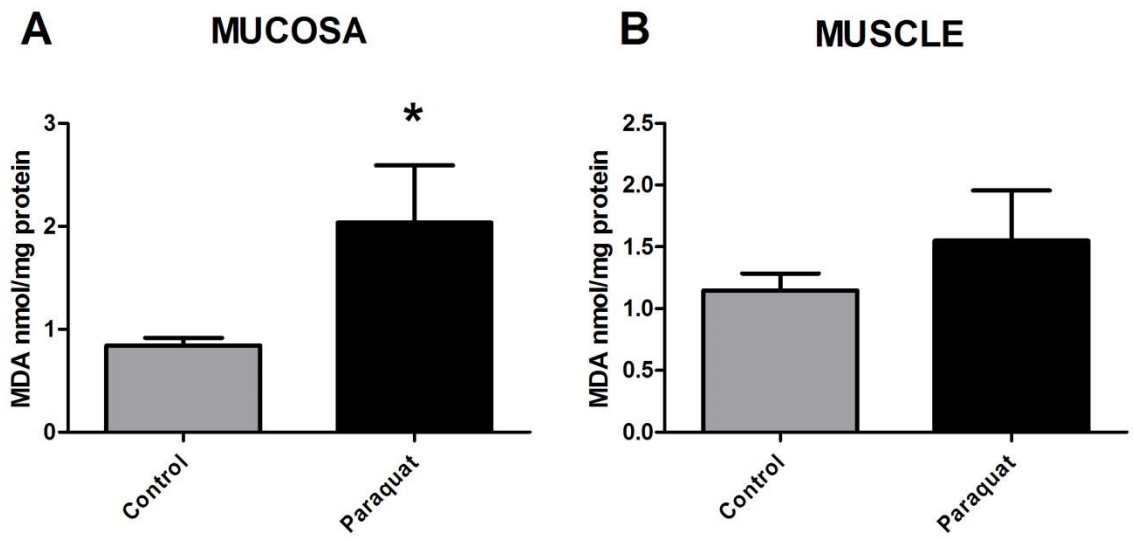
BN2 4GJ

Tel: +44 (0)1273 642078

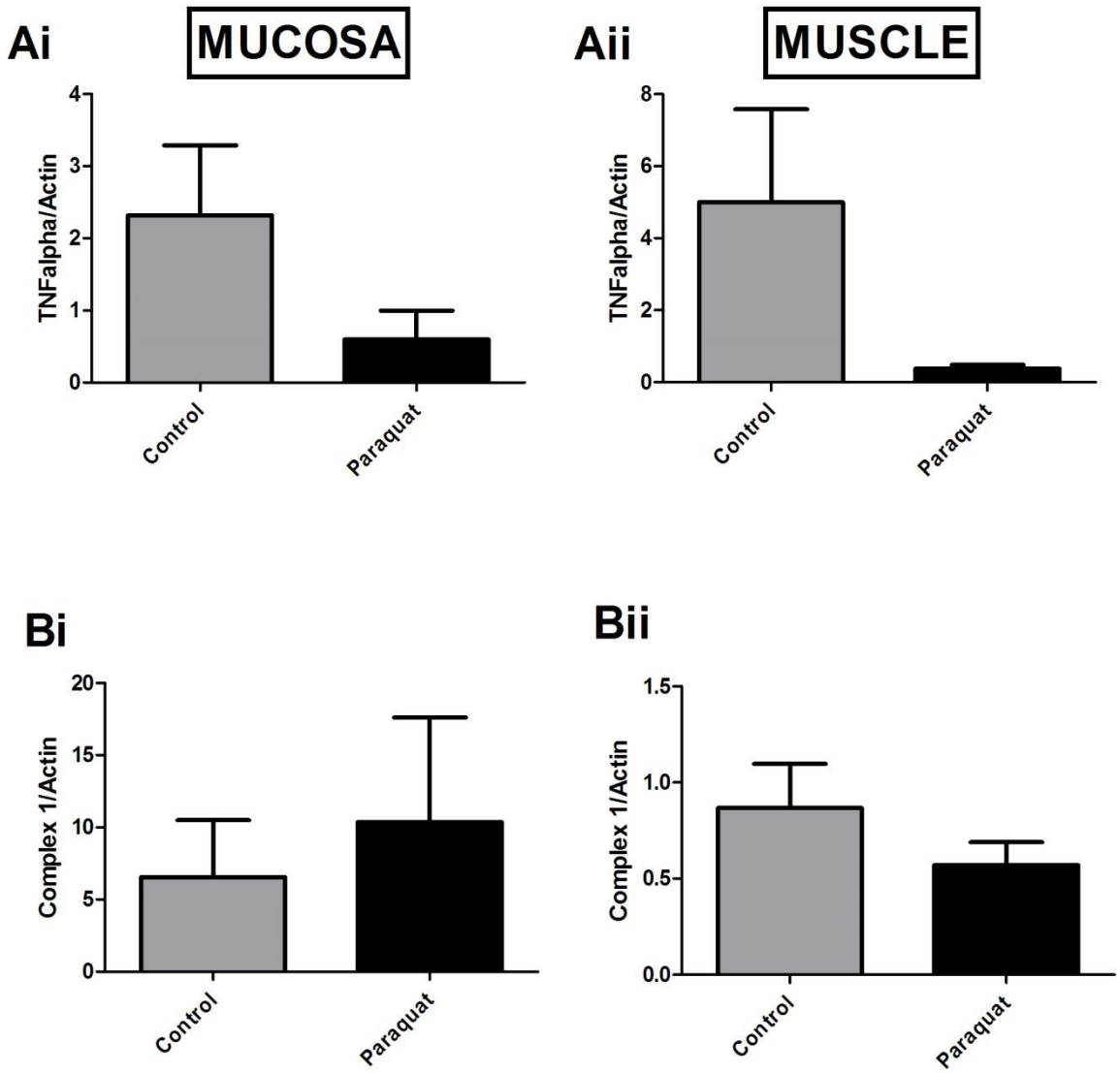
E-mail: m.s.yeoman@brighton.ac.uk.

608

Supplementary Figure 1: Paraquat increases MDA levels in the mucosa but not distal colon smooth muscle. Bar graphs showing how MDA levels in both the mucosa (A) and smooth muscle (B) are affected by paraquat treatment. N=5 for each group; * $p<0.05$.



Supplementary Figure 2: Paraquat does not alter expression of TNFalpha or complex 1 in the distal colon. Bar graphs illustrating the change in expression of TNFalpha (Ai, Aii), complex 1 (Bi, Bii). N=5 per group.



617

618

619

620

621