

Alterations in Intestinal Contractility during Inflammation Are Caused by Both Smooth Muscle Damage and Specific Receptor-mediated Mechanisms

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Aim To evaluate motoric intestinal disturbances during inflammation with *Trichinella spiralis* in rats as an experimental model.

Methods We examined the changes in worm-positive (jejunum) and worm-free (ileum) intestinal segments of rats infected with *T. spiralis*. To investigate the relationship between structural and functional changes in smooth muscle, we measured the thickness of the muscle layers of rat jejunum and ileum. Mechanical responses to KCl 30 mmol/L, acetylcholine (ACh) 10^{-8} - 10^{-4} mol/L, substance P (SP) 10^{-9} - 10^{-5} mol/L, and to electrical field stimulation of longitudinal muscle strips in the jejunum and ileum were studied in muscle bath as controls (day 0) and on day 2, 6, 14, 23, and 72 after infection.

Results After *T. spiralis* infestation, an inflammation of the mucosal and submucosal layers of jejunum was observed, whereas in the worm-free ileum there was not any inflammatory infiltrate. Increase in the smooth muscle thickness of both jejunum and ileum were correlated with increased responses to depolarizing agent KCl and to ACh. However, responses to SP were decreased on day 14-23 after infection in jejunum and from day 6-14 after infection in ileum. Electric field stimulation-induced contractions were transiently decreased in the jejunum (day 2 after infection) but in the ileum the contractile responses were decreased until the end of the study period.

Conclusions Alterations in intestinal smooth muscle function do not require the presence of the parasite and the absence of histopathological signs of inflammation do not warrant intact motor function. Changes in motor responses after *T. spiralis* infection are not only due to smooth muscle damage but also to disturbances in specific receptor-mediated mechanisms.

Numerous clinical studies have reported that altered gastrointestinal motility, cramps, and diarrhea are common features following a wide variety of intestinal inflammations. Frequently, such motor changes persist unexpectedly long after the recovery of the normal structure of the mucosa, and in some instances they affect not only the inflamed area, but also remote regions of the gastrointestinal tract (1,2).

The high incidence of motor disturbances during intestinal inflammation has prompted intensive research aiming to set up experimental models useful to study such disorders. The reported changes in muscle function are similar regardless of the model used to induce inflammation, ie, *T. spiralis* infection or administration of different chemical compounds as trinitrobenzenesulfonic acid (TNBS), mitomycin, and acetic acid (3). Changes that occur during the intestinal phase of infection with *T. spiralis* in the rat have been well studied. The nematode *T. spiralis* is an intraepithelial parasite that preferentially inhabits the proximal small intestine of rats (4). In the rat, *T. spiralis* infection is a relatively mild and long-lasting inflammatory process during which the only remarkable symptoms are a decrease in food intake and body weight. In this model, survival of the animals is warranted for long periods and the inflammation induced by the parasite, though noticeable, is moderate in comparison with that induced by chemical compounds such as TNBS acid. When *T. spiralis* larvae are given orally to the animals, the parasite inhabits the duodenum and the jejunum but it does not reach the ileum (4). Thus, in our study, specimens of jejunum were taken as representative of inflamed worm-positive and specimens of ileum were considered representative of non-inflamed worm-free segments. In the enteric phase of the infection, the presence of adult worms and larvae in the mucosal and submucosal layers causes an inflammatory response and functional changes in the motility of small intestine (5-7).

In vivo, myoelectric activity is altered (5) and the rate of intestinal transit is increased (6), which might be viewed as an extension of the immune response leading to the expulsion of the parasite. In vitro, contractility of the smooth muscle is increased (7) and the function of certain enteric nerves is depressed (8). In addition, the thickness of the muscle layers is increased due to both hypertrophy and hyperplasia, which may additionally alter gastrointestinal motility, since increased smooth muscle mass may exacerbate muscle contraction and amplify the effect of excitatory stimuli (9). Changes in propulsive intestinal activity were also observed in denervated gut segments, suggesting that elements intrinsic to the intestinal wall, ie, enteric nerves and smooth muscle cells, play a crucial role (10). Many observed functional and morphological changes in the worm-free segments of the ileum of *T. spiralis*-infected rats (11,12) indicate that the local presence of the parasite is not required for the systemic response and the motor changes are not restricted to the site of inflammation. Specific changes have also been reported in contractility, which were not related to the responses to depolarizing agents but to specific agonists, such as motilin and acetylcholine (13).

Our aim was to determine the time-course of morphological (inflammation and hypertrophy) and contractile changes in the intestine at different times after infection, using healthy controls and *T. spiralis* infected rats. We also correlated structural changes with functional ones and compared worm-positive inflamed (jejunum) against worm-free non-inflamed (ileum) areas of the rat small intestine, using KCl, acetylcholine (ACh), substance P (SP) as stimuli and contraction elicited by electrically field stimulation.

Materials and methods

Trichinella spiralis larvae preparation

The larvae of *T. spiralis* were obtained from CDI mice infected 30-90 days earlier, according to the

method described by Castro and Fairbairn (14). Briefly, larvae were isolated from the mouse skeletal muscle by digestion with the standard solution containing 1% pepsin and 1% HCl. Rats were infected by oral administration of 7.500 *T. Spiralis* larvae suspended in 1 mL of 0.9% NaCl solution.

Animal model of *Trichinella spiralis* infection

We used male Sprague-Dawley rats (300-350 g), 8-10 weeks old, kept at a constant temperature (22-23°C) and with lighting cycle of 12 hours light/12 hours dark. The day before the experiment, animals were fasted overnight but allowed *ad libitum* access to water. Rats were euthanized on days 2, 6, 14, 23, and 72 after infection by decapitation. After exsanguination, the abdomen was immediately opened and 2 cm segments of median ileum and jejunum (5 cm distal to the ligament of Treitz) were removed. The tissue was placed in previously bubbled (95% O₂ and 5% CO₂) Krebs solution. The Ethical Committee of the Universitat Autònoma de Barcelona approved the procedure.

Histopathological study

Samples of the jejunum and ileum were taken from control and *T. Spiralis*-infected rats on days 2, 6, 14, 23, and 72 after infection (n=6). The samples were processed for histopathology, stained with hematoxylin and eosin, and finally viewed under microscope (×400). A standard scoring based on inflammatory cell infiltration was used to evaluate the inflammatory process. The level of inflammation was classified as no infiltration (-), mild (+), moderate (++) and severe, intense infiltration (+++), depending on the extent of inflammatory cell infiltration at mucosal, submucosal, and muscular level. The same samples were also used to measure the thickness of both circular and longitudinal muscle layers. Individual data of thickness were obtained from the average value of four measurements per preparation made with an ocular

micrometer. All observations were performed in a simple blind protocol.

Muscle bath experiments

Whole full thickness segments of the ileum or jejunum were placed in longitudinal direction in a 10 mL muscle bath, filled with pre-aerated Krebs solution at 37°C. Krebs solution contained (in mmol/L) 115.48 NaCl, 4.61 KCl, 2.5 CaCl₂, 1.16 MgSO₄, 1.14 NaH₂PO₄, 21.9 NaHCO₃, 10.09 glucose, and pH 7.4. Atropine, ACh, SP, and phentolamine; and sodium nitroprusside, tetrodotoxin, and propranolol-HCl (RBI, Natick, MA, USA) were dissolved in distilled water to make stock solutions. Sodium nitroprusside was prepared as aqueous solution immediately before use. The volume that was added to the bath never exceeded 5% of its total volume.

The upper end of the preparation was tied to an isometric transducer (Harvard UF-1, SDR Clinical Technology, Sydney, Australia) and preloaded with 1.0-1.5 g. Tissue was allowed to equilibrate for 1 hour until a stable baseline was attained. Data corresponding to mechanical activity were displayed, analyzed, and stored in a computer by use of Datawin-2 software (Panlab, Barcelona, Spain), coupled to an ISC-16 A/D card (25 samples/s). At the start of each experiment, KCl 30 mmol/L (Sigma, St. Louis, MO, USA) was added to the bath and this contraction was considered a reference response (RR). At the end of the experiment, the response to KCl 30 mmol/L was measured again to assess preparation responsiveness. The amplitude of contractions corresponding to cumulative concentration-response curves for ACh 10⁻⁸-10⁻⁴ mol/L (Sigma) and SP 10⁻⁹-10⁻⁵ mol/L (Sigma) and to electric field stimulation-induced responses were expressed both in grams and as a percent of the initial KCl reference response (%RR).

Data were fitted to a Michaelis-Menten equation by use of nonlinear regression. The equation was used to estimate maximal effect (E_{max}) and pD₂ (negative logarithm of the concentration

that induces 50% of the maximal contraction). E_{\max} and pD_2 obtained on days 2, 6, 14, 23, and 72 after infection were compared to control tissue.

Responses to electrical field stimulation

Electrical field stimulation was performed through platinum electrodes connected to a pulse generator (Harvard double channel stimulator). Excitatory responses were obtained by application of repetitive stimuli (10 Hz, 0.3 ms, 40 V) with duration of 15 seconds in both control and infected tissues. Peak responses were calculated as percentage of the KCl (%RR). The responses elicited by electrical stimulation were also measured in the presence of the nitric oxide donor sodium nitroprusside 10^{-3} mol/L (5 minutes after its addition to the bath solution) and in non-adrenergic non-cholinergic conditions (atropine, phentolamine, and propranolol, each at 10^{-6} mol/L added 10-minute before). In each case, the amplitude of the response obtained on days 2, 6, 14, 23, and 72 after infection were compared to control tissue.

Statistical analysis

All data were presented as mean \pm standard error of mean (SEM). The number of repetitions (n) represented the number of experiments performed with samples taken from different animals. ANOVA followed by Bonferroni post-hoc test was used to compare data obtained on days 2, 6, 14, 23, and 72 after infection with data obtained from control tissue. The correlation was determined between structural (hypertrophy) and functional changes (E_{\max} values obtained from KCl and ACh and SP concentration-re-

sponse curves). Statistical analysis was performed with GraphPad InStat software, version 3.06 (San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

Results

A significant decrease in food intake and body weight was observed during the first 2 weeks after infection. Loss in body weight averaged 1.2% per day 2-12 days after infection. After day 12, both parameters were back to normal.

Histopathological study

T. Spiralis infection induced an intense inflammatory response with mixed infiltrate of neutrophil and eosinophil cells in the jejunum. Cell infiltration affected the mucosal and submucosal layers of the jejunum from day 2 after infection until day 23 after infection, with no signs of inflammation in the muscular layers. Severe inflammation scores were observed between 2 and 14 days after infection and a mild inflammation was present on day 23 after infection (Table 1). In contrast, no signs of inflammation were seen in the worm-free ileum throughout the experimental period.

T. Spiralis infection provokes trophic changes in the circular and longitudinal smooth muscle layers of both jejunum and ileum. Increased thickness of jejunal and ileal muscle layers was noted from day 2 to day 72 after infection, with the most prominent changes found in samples from days 14 and 23 after infection (Figure 1). Throughout the experimental period, we did not note any increase in the intercellular space.

Responses to KCl, acetylcholine, and substance P

The amplitude of KCl-induced response at the start and end of each experiment was similar. In *T. Spiralis* infected rats, increased contractile responses to KCl were noted in both jejunum and ileum from day 6 to day 23 after infection (Figure 2). On day 23 after infection, it was increased for

Table 1. Severity of mucosal inflammation estimated* according to neutrophil and eosinophil cell infiltration after infection with *Trichinella spiralis*

Sample tissue	Days after infection					
	control (0)	2	6	14	23	72
Jejunum	-	+++	+++	+++	+	-
Ileum	-	-	-	-	-	-

*Values are expressed according to cell infiltration in 7 animals as follows: no infiltration (-), mild (+), moderate (++), and severe infiltration (+++).

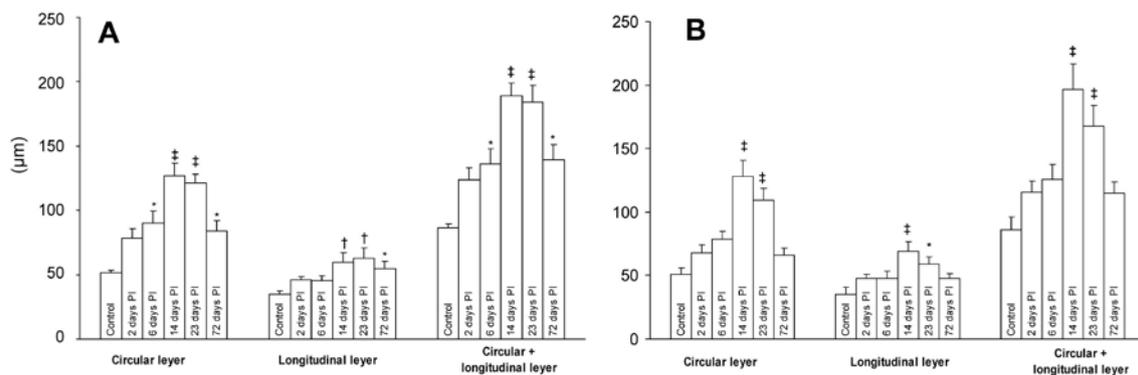


Figure 1. Thickness of longitudinal and circular muscle layer of jejunum (A) and ileum (B) under controlled conditions and at different times after infection. Data collected from 6 animals are expressed as means±SEM (µm); **P*<0.05, †*P*<0.01, ‡*P*<0.001 indicate differences between healthy and *Trichinella spiralis*-infected rats.

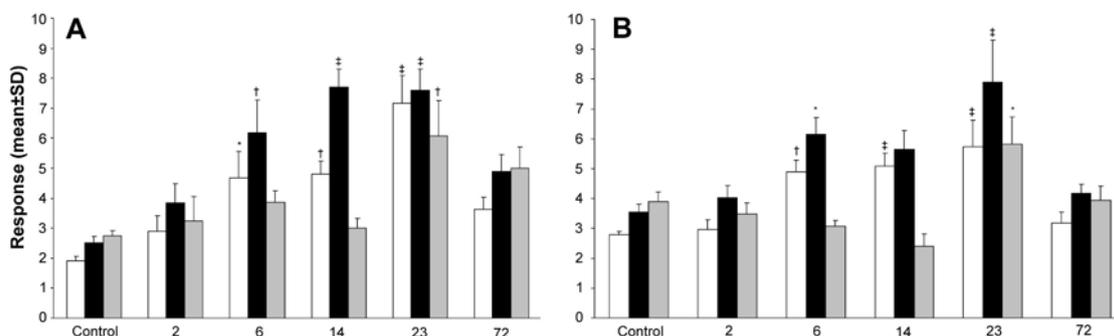


Figure 2. Responses of the jejunum (A) and ileum (B) to 30 mmol/L KCl-induced contraction (open column), E_{max} (g) response to acetylcholine (ACh; closed column) and E_{max} (g) response to substance P (SP; gray column) in healthy (control) and *T. spiralis*-infected rats. Data collected from 7 animals are expressed as a mean±SEM (g). **P*<0.05, †*P*<0.01, and ‡*P*<0.001 indicate differences between healthy and *Trichinella spiralis*-infected rats.

more than 2-fold in both tissues. A similar time-course in the responses to ACh was observed (Figure 2). An increase in the E_{max} response to ACh was observed between 6 and 23 after infection in the jejunum and on days 6 and 23 after infection in the ileum. At the end of the study period (72 days after infection), the E_{max} value of ACh was partly recovered. A different time-course was observed in the response to SP, indicated by the fact that E_{max} response to SP was increased only on day 23 after infection (Figure 2).

To correlate morphological and functional changes, we plotted smooth muscle thickness against the E_{max} values to KCl, ACh, and SP. A significant linear correlation was found between KCl response and muscle thickness both in the jejunum ($r^2=0.74$; $P=0.028$) and in the ileum ($r^2=0.67$; $P=0.044$) (Figure 3). Similarly, the

E_{max} value to ACh also showed linear correlation with muscle thickness in the jejunum ($r^2=0.92$; $P=0.002$) and ileum ($r^2=0.81$; $P=0.014$). In contrast, no linear correlation was observed between E_{max} value in response to SP and muscle thickness in the jejunum ($r^2=0.25$; $P=0.310$) and ileum ($r^2=0.008$; $P=0.859$) (Figure 3).

Because of the correlation between smooth muscle thickness and KCl E_{max} value, we normalized our data using KCl as a reference response (%RR). The E_{max} values of ACh, expressed in percentage of KCl-induced reference response (%RR), showed no significant changes throughout the study period (Figure 4). The pD_2 value was decreased for jejunal samples on days 6 and 14 after infection, while pD_2 values of ileal samples were not significantly changed (Figure 4).

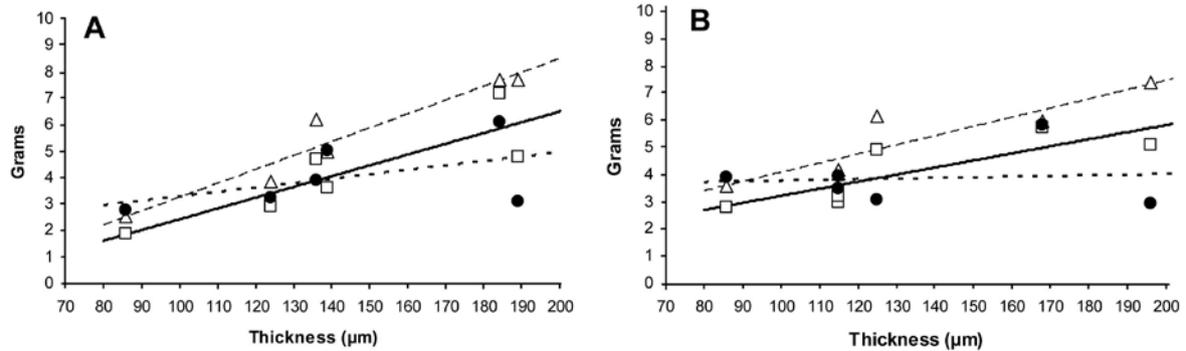


Figure 3. Correlation between full muscle thickness (μm) and KCl (solid line with square symbols), acetylcholine (ACh; dashed line with triangle symbols) and substance P (SP; dotted line with circle symbols) response (g) of the jejunum (A), and the ileum (B). Correlation is present between muscle thickness, KCl and ACh response, and absent in case of SP.

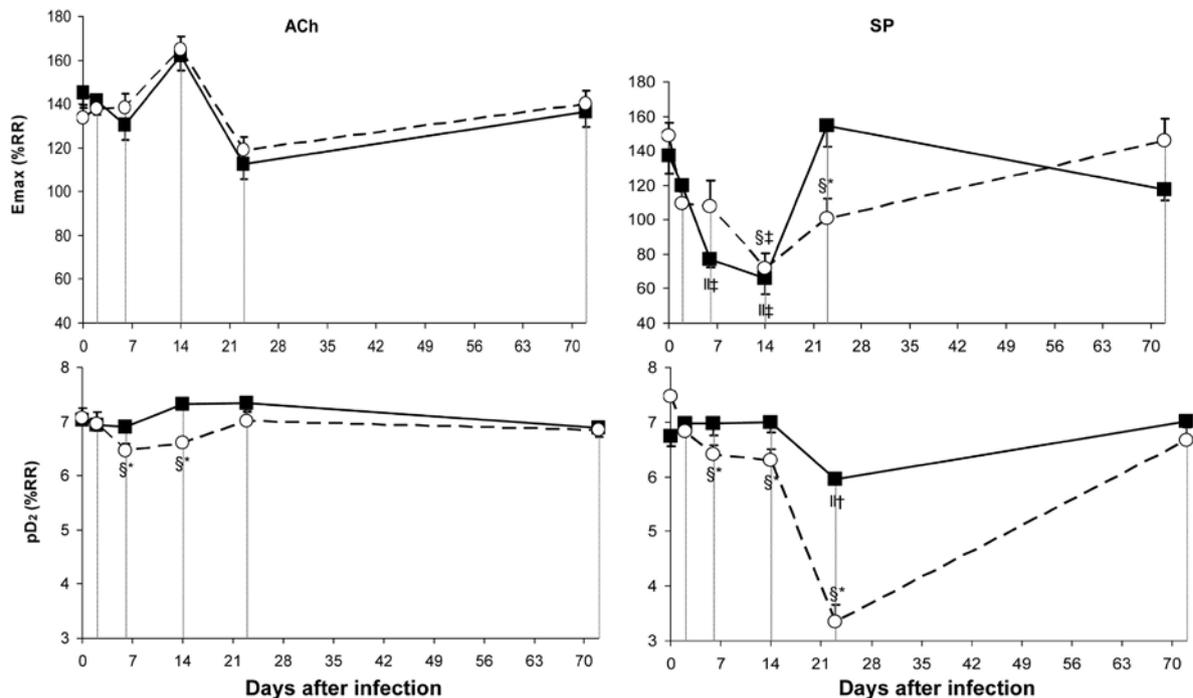


Figure 4. Time-course of responses to acetylcholine (ACh) and substance P (SP) with KCl as a reference response (%RR) from the jejunum (dashed lines with circle symbols) and ileum (solid lines with square symbols). Upper panels show the changes in E_{max} values, expressed in percentage of KCl-induced reference response (%RR), and lower panels show the evolution of pD_2 , induced by ACh and SP. Data collected on 7 animals are presented as mean \pm SEM. * P <0.05, † P <0.01, and ‡ P <0.001 indicate differences between healthy (control) and *Trichinella spiralis*-infected rats.

With respect to the response to SP (%RR), a significant decrease in E_{max} values was found on days 14 and 23 after infection for jejunal samples and on days 6 and 14 after infection for ileal preparations. Significant decrease in pD_2 values on days 6, 14, and 23 after infection for jejunal tissue and on day 23 after infection for ileal tis-

sue indicated that both tissues were less sensitive to SP (Figure 4).

Response to electrical field stimulation

Application of electrical stimuli (10 Hz, 0.3 ms, 40 V, and 15 s) resulted in a transient relaxation

followed by a contraction in the jejunum and ileum, both being tetrodotoxin-sensitive. The contraction averaged 70.8 ± 4.8 %RR for the jejunum and 77.7 ± 6.6 %RR for the ileum (Figure 5). The response of the jejunum was decreased on day 2 after infection, but later the contraction returned to normal values. The incubation with the nitric oxide donor sodium nitroprusside 10^{-3} mol did not modify the responses to electrical field stimulation neither in controls nor in tissue form infected rats at any day after infection (data not shown). The non-adrenergic non-cholinergic component of the jejunal response to electrical field stimulation remained unchanged (Figure 5). In the ileum, the responses to electrical field stimulation were decreased starting from day 2 after infection. The non-adrenergic non-cholinergic contractile component of ileal response to electrical field stimulation was also significantly decreased from day 6 to day 23 after infection (Figure 5).

Discussion

In *T. spiralis*-infected rats, histopathological signs of inflammation in the mucosal and submucosal layers of the jejunum were well correlated with the presence of parasites. Nevertheless, muscle thickness of the longitudinal and the circular layer was increased from day 6 until day 72 after in-

fection in both inflamed and non-inflamed areas. This indicates that an inflammation, primarily located in the mucosa and submucosa, has a powerful mitogenic effect on the underlying smooth muscle. Blennerhassett et al (9) also reported that hyperplasia and hypertrophy associated with *T. spiralis* infection were responsible for increased smooth muscle thickness of rat small intestine. Other studies reported that presence of edema, production of extracellular matrix, or increases in collagen did not contribute to the apparent increase in tissue mass (9,15).

Contractility results from the ileum and jejunum showed that both tissues were similarly affected, suggesting that local inflammation is not the causal factor of these changes. The contractile responses to KCl and ACh were increased from day 6-23 after infection, whereas an increase in SP response was observed only on day 23 after infection in both tissues. It was reported that in rats, in which colitis had been induced by intrarectal administration of *T. spiralis* larvae, the motility of the non-inflamed ileum was decreased (12). Taken together, these data suggest that impaired function in non-inflamed areas is a generalized feature and the host response rather than local mechanisms seem to be responsible for the structural and functional changes observed in the worm-free ileal segments. The increased propulsive activity might be also viewed as an adaptive

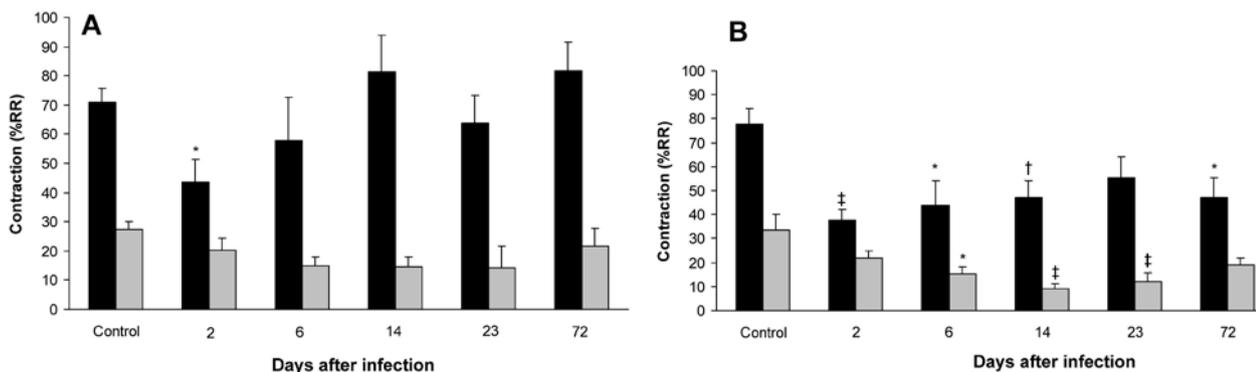


Figure 5. Contractile responses of the jejunum (A) and ileum (B) preparations to electrical field stimulation (40V, 10 Hz, 0.3 ms, 15 s) under controlled (closed bars) or non-adrenergic non-cholinergic conditions (NANC; open bars) by addition of atropine, phentolamine, and propranolol, each at 10^{-6} mol/L, added 10 minutes earlier. Data collected from 7 animals are presented as mean \pm SEM and expressed in percentage of 30 mmol/L KCl-induced reference response (%RR). * $P < 0.05$, † $P < 0.01$, and ‡ $P < 0.001$ indicate differences between healthy (control) and *Trichinella spiralis*-infected rats.

change associated with the host immune system, enabling it to act as an “extension” of the immune system in removing the parasite from the intestine (6). Additionally, intestinal inflammation may result in an increase in actin synthesis in smooth muscle, so we cannot exclude that some of the changes observed could result from increased contractile protein content (15).

Since the contractile capacity of the muscle layer is directly proportional to the muscle mass, we tried to discriminate motor changes related to muscle thickness from those related with alterations at the receptor level. Different methods to normalize the contractile responses for the amount of muscle mass are used in the literature, which may lead to conflicting results (16). We found that changes in thickness of muscle layers correlated with KC- and ACh-induced contractions both in the jejunum and ileum, suggesting that the increase in contractility induced by KCl and ACh might be a consequence of the hypertrophy and/or hyperplasia. In contrast, we did not observe such a correlation between SP response and muscle thickness. In view of the relationship found between muscle thickness and response to KCl, we used the response to KCl as a reference response to normalize contractility data. We did not find significant changes in E_{\max} values in the ACh response neither in the jejunum nor in the ileum. In contrast, E_{\max} values obtained with SP were significantly decreased on days 14 and 23 after infection for jejunal samples and on days 6 and 14 after infection for ileal samples. This effect might be due to an increased release of SP or an altered sensitivity to SP during *T. spiralis* infection (17,18). Alternatively, conformational changes involved in the interaction between the SP receptors and its effector system may also be affected.

The contractile response induced by electrical field stimulation is mainly due to ACh and SP release from enteric motor neurons. In the jejunum, the response to electrical field stimula-

tion is transiently decreased on day 2 after infection, reaching normal values between 6 and 72 days after infection. However, there was a pronounced decrease in contractile responses to electrical field stimulation in non-inflamed ileum throughout the study period. This might be due to pre-junctional mechanisms, since the response to exogenously added ACh was not apparently altered. Additionally, a decrease in the response to exogenously added SP may contribute to the decreased response to electrical field stimulation observed in non-adrenergic non-cholinergic conditions. Similarly, electrical field stimulation-induced relaxations of jejunum and ileum from *T. spiralis* infected rats caused a significantly decreased relaxation only in the ileum on days 14-23 PI, whereas jejunal strips did not display any significant change during the 72 days of study period (19).

In some preparations, incubation with NO donors may result in a prejunctional inhibition of NO synthase activity, acting as a feedback regulation of nitrenergic transmitter release (20). Baccari et al (21) also showed that sodium nitroprusside was able to depress the electrical field stimulation-elicited cholinergic contractions, suggesting a putative neuro-modulatory role for NO. However, we have not found any evidence supporting the existence of such a mechanism in the rat jejunum or ileum.

In conclusion, our work shows that an impaired neural function or long-lasting damage of the excitation-contraction coupling might be the cause of altered motility in the non-inflamed ileum of *T. spiralis*-infected rats.

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