Title page

Effects of melatonin on gallbladder neuromuscular function in acute cholecystitis

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Running Title Page

Running title: Melatonin in acute cholecystitis

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Number of text pages: 28
Number of tables: 1
Number of figures: 7
Number of references: 40
Number of words in the Abstract: 229
Number of words in the Introduction: 433
Number of words in the Discussion: 1249

Abbreviations:

EFS, electrical field stimulation; NO, nitric oxide; ACh, acetylcholine; AC, acute acalculous cholecystitis; CBDL, common bile duct ligation; mN, milliNewtons; DL, deligation; TTX, tetrodotoxin; L-NAME, No-nitro-L-arginine methyl ester; NANC, non adrenergic non cholinergic (NANC)
ABSTRACT

Gallbladder stasis is associated to experimental acute cholecystisitis. Impaired contractility could be, at least in part, the result of inflammation-induced alterations in the neuromuscular function. This study was designed to determine the changes on gallbladder neurotransmission evoked by acute inflammation and to evaluate the protective and therapeutic effects of melatonin. Experimental acute cholecystitis was induced in guinea pigs by common bile duct ligation for two days and then, the neuromuscular function was evaluated using electrical field stimulation (EFS, 5–40 Hz). In a group of animals with the bile-duct ligated for two days, a de-ligation of the duct was performed and after two days the neuromuscular function was studied. The EFS-evoked isometric gallbladder contraction was significantly lower in cholecystitic tissue. In addition, inflammation changed the pharmacological profile of these contractions that were insensitive to tetrodotoxin but sensitive to atropine and ω-conotoxin, indicating that acute cholecystitis affects action potential propagation in the intrinsic nerves. Nitric oxide (NO)-mediated neurotransmission was reduced by inflammation which also increased the reactivity of sensitive fibers. Melatonin treatment prevented qualitative changes in gallbladder neurotransmission, but did not improve EFS-induced contractility. The hormone recovered gallbladder neuromuscular function once the biliary obstruction was resolved even when the treatment was started after the onset of gallbladder inflammation. These findings show for the first time the therapeutic potential of melatonin in the recovery of gallbladder neuromuscular function during acute cholecystitis.
INTRODUCTION

Gallbladder tone is mainly regulated by both myogenic mechanisms and neuro-hormonal inputs. The neural control of gallbladder motility involves reflexes that include both efferent and afferent nerve fibers as well as the intrinsic plexus in the gallbladder wall (Mawe et al., 2006). Acetylcholine (ACh) released from cholinergic neurons induces contraction of the gallbladder smooth muscle through muscarinic receptors (Parkman et al., 1999b) and has neuromodulatory functions promoting or inhibiting the release of other neurotransmitters (Parkman et al., 1999b). Cholinergic neurons co-express other neurotransmitters such as NO and several neuropeptides (Talmage et al., 1992). Afferent nerve fibers containing calcitonin gene-related peptide and tachykininins have also been described in the ganglionated plexus of the gallbladder (Mawe and Gershon, 1989).

Acute acalculous cholecystitis (AC) is characterized by gallbladder inflammation in the absence of gallstones. Although its pathogenesis is unknown, gallbladder stasis is always present, probably as the result of the deleterious neural and muscular actions of inflammatory mediators such as reactive oxygen species and prostaglandins (Pozo et al., 2004). In animal models, it has been described that cholecystitis reduces gallbladder contractile responses to agonists that act directly on smooth muscle cells (Parkman et al., 1999a; Xiao et al., 2001) and also causes alterations in calcium signalling and contractile machinery (Gomez-Pinilla et al., 2006b). In addition, EFS-induced contractions are also impaired in inflamed gallbladder, mainly due to the reduction in the function of cholinergic nerves and the up-regulation of the inhibitory nitrergic component (Parkman et al., 2000). The effect of cholecystitis on afferent fibers has not yet been explored.
Melatonin, the main product of pineal gland, is a potent free radical scavenger and activates a broad group of antioxidant cellular mechanisms (Tan et al., 2002). These properties made melatonin efficacious against different diseases where oxidative stress is the main cause (Karasek, 2004). The gastrointestinal tract is an important source of melatonin (Kvetnoy et al., 2002). The liver and the gallbladder are especially exposed to high levels of the hormone since hepatic metabolism is the major pathway for deactivation of melatonin, (Lane and Moss, 1985) which is also present in active form in bile and concentrated in the gallbladder (Tan et al., 1999). In the gastrointestinal tract, melatonin has a gastroprotective function (Konturek et al., 1997) and therapeutic effects against malignance-associated to irritable bowel syndrome (Head and Jurenka, 2003).

The aims of this study were to investigate the effects of acute cholecystitis in the neuromuscular transmission and to evaluate the impact of melatonin treatment. Our results indicate that melatonin restores neuromuscular function in inflamed gallbladder, which can be of importance to recover gallbladder contractility in this pathological condition.

**Methods**

**Design-animal preparations**

Male guinea pigs, weighing 400 g to 600 g, were used in the study. Acute acalculous cholecystitis(AC) was induced to animals by common bile duct ligation (CBDL) for 2 days, as previously described (Gomez-Pinilla et al., 2006b). This method was approved by the Animal Care and Ethical Committees of the University of Extremadura. In brief, after anesthesia with ketamine hydrochloride (20 mg/Kg ip) and xylacine (5 mg/Kg ip) a laparotomy was performed and the distal end of the common bile duct was ligated. Two days after, the animals were sacrificed for tissue harvest (n = 28).
used in this study, the gallbladder is stretched as the result of bile duct ligation and the continuous bile output. Taking this into account, it would be difficult to see any improvement in the neuromuscular function keeping the bile duct ligated, which represents a remarkably extreme pathological condition. To solve this, in a group of animals, two days later CBDL the common bile duct was de-ligated (DL) under anaesthesia with microsurgical scissors and two days after the animals were sacrificed (n = 28). For both experimental models, a group of guinea pigs were sham operated (n = 4), which included all the surgical steps except the common bile duct ligation.

**Melatonin and tempol administration**

Guinea pigs were treated orally with melatonin (2.5 or 30 mg/Kg/day). Melatonin was dissolved in glucose solution (1.5 %) and placed in the oropharynx by a syringe. This treatment was applied daily at the same time, just before the light in the animal house was switched off (7:00 PM). Melatonin was administered 14 days before the sacrifice of the animals in both experimental groups, AC and DL. In a group of animals subjected to DL melatonin treatment (30mg/kg) started 12 hours after CBDL was performed and continued until the sacrifice of the animal. Tempol was administered in the drinking water at 1mmol/L for 14 days prior to the animal sacrifice.

**Functional studies**

At the appropriated time, the animals were killed with deep halothane anaesthesia and cervical dislocation. Gallbladders were removed and immediately placed in cold Krebs-Henseleit solution (for composition, see *Solutions and drugs*) at pH 7.35. The gallbladder was cut in longitudinal full thickness strips (3 x 10 mm) that were placed vertically in a 10-ml organ bath filled with Krebs-Henseleit solution maintained at 37°C and gassed with 95% O₂-5% CO₂. Isometric contractions were measured using force displacement transducers that were interfaced with a Macintosh computer using a
MacLab hardware unit and software (ADInstruments, Colorado Springs, CO). The muscle strips were placed under an initial resting tension equivalent to a 1.5-g load. Intrinsic nerves were activated by EFS with a pair of external platinum ring electrodes connected to a square-wave stimulator (Cibertec CS9/3BO). Trains of stimuli (0.3-ms duration, 5–40 Hz, 350-mA current strength) were delivered for 10 s at 3-min intervals. After construction of a frequency-response curve and in order to pharmacologically characterize the neurogenic responses, antagonists/inhibitors were added to the organ bath for 20 min, and then the EFS protocols were repeated.

**Malondialdehyde (MDA) and reduced glutathione (GSH) assays**

Gallbladder fragments of about 10 mg were emplaced in a cold phosphate buffer at a proportion 1/5 (weigh/volume), homogenized with an homogenizer (Ika-Werke, Staufen, Germany) for two minutes and centrifuged at 10000 rpm for 15 minutes at 4°C. The protein concentration was then quantified with a commercial kit (TPRO-562, Sigma) and the rest of homogenate was treated with cold perchloric acid (7% vol/vol) to eliminate proteins and kept at -80°C until analysis. Malondialdehyde (MDA) level, an index of lipidic peroxidation, was determined based on colorimetric Recknagel’s method (Waller and Recknagel, 1977). Briefly, the samples were incubated with 0.4% of thiobarbituric acid at 80°C for 20 minutes and later the sample absorbance at 550 nm was measured. Reduced glutathione determination was carried out following the Hissin and Hilf method (Hissin and Hilf, 1976): samples were incubated with 0.005% of orthophtaldehyde in the darkness at room temperature for 45 minutes and the fluorescent complex formed, indicative of reduced glutathione (GSH) level, was measured with a fluorimeter (excitation 350 nm, emission 425 nm).

**Solutions and Drugs.** The Krebs-Henseleit solution contained 113 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, and 11.5 mM
D-glucose. This solution had a final pH of 7.35 after equilibration with 95% O₂-5% CO₂. The phosphate buffer used to homogenize the tissue contained (in mM): NaCl 20, KCl 2.7, Na₂HPO₄ 16, NaH₂PO₄ 4, pH 7.4. Drug concentrations are expressed as final bath concentrations of active species. Drugs and chemicals were obtained from the following sources: atropine, L-NAME, melatonin and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl or 4-hydroxy-tempo) were from Sigma Chemical Company (St. Louis, MO); ω-conotoxin GVIA, E-capsaicin and tetrodotoxin citrate were from Tocris Cookson (Bristol, UK). Ketamine was from Merial (Lyon, France). Xylacine was supplied by Bayer (Kiel, Germany). Other chemicals used were of analytical grade from Panreac (Barcelona, Spain). Stock solutions of atropine, capsaicin and ω-conotoxin GVIA were prepared in DMSO. The solutions were diluted such that the final concentration of DMSO was ≤ 0.1% v/v. This concentration of DMSO did not have effects on gallbladder tone.

**Data analysis.** Results are expressed as means ± S.E.M. of n gallbladder strips from at least 5 different animals. Gallbladder tension is given in millinewtons per milligrams of tissue (mN/mg). Statistical differences between multiple groups or the effects of inhibitor treatments were tested using appropriate analysis of variance (ANOVA). Differences were considered significant at $P < 0.05$.

**Results**

**Effects of acute cholecystitis on gallbladder neuromuscular function.**

EFS was used to stimulate the neuronal network in the gallbladder wall and the recording of isometric tension allowed us to evaluate the neuromuscular function. EFS evoked a frequency-dependent contraction in control strips that was significantly decreased in animals subjected to CBDL compared with sham controls (Fig 1&B). The
diminished response was reflected by reductions in both the amplitude and the duration of the contractions ($P < 0.01$ and $P < 0.001$, two-way ANOVA for both, Fig. 1C & D).

In order to determine the neural and myogenic components of the EFS-evoked contractions, the nerve Na$^+$ channel inhibitor, tetrodotoxin (TTX) was used. In control strips, 1 µM of TTX abolished EFS-elicited responses (Fig. 2A). In inflamed strips, tetrodotoxin was not effective (3.6 % of enhancement at 25 Hz) (Figure 2B), but when the strips were co-incubated with tetrodotoxin (1 µM) plus ω-conotoxin GVIA (0.1 µM), a N-type calcium channel blocker, there was a reduction (63 % of inhibition at 25 Hz) in the contractile response evoked by EFS (Fig. 2B). These results indicate that in inflamed gallbladder the transmission of the action potential along neural fibers is impaired and EFS stimulates neurotransmitter release directly from nervous terminals.

To elucidate the neurotransmitters involved in the EFS-induced contraction we tested several antagonists/inhibitors on this neural response. In control animals, we found that 1 µM atropine reduced the EFS-elicited contractile response (82 % inhibition at 25 Hz) (Fig. 3A) but the strips from cholecystitic animals were less sensitive to atropine blockade (30 % of inhibition at 25Hz, Fig. 3D). The impact of inflammation on the contribution of NO was tested by using the inhibitor of nitric oxide synthase, L-NAME (100 µM). This inhibitor enhanced EFS-induced contraction in strips from control guinea pigs, especially at the lowest frequencies assayed (90 % of enhancement at 5 Hz, Fig. 3B) but had little effect in inflamed strips (17 % of enhancement at 5 Hz; Fig. 3E).

Small diameter sensory neurons that are sensitive to capsaicin play a major role in the generation of neurogenic inflammation (Sann et al., 1996). When we induced sensory nerve desensitization by the treatment with a high concentration of capsaicin (10 µM) we found no effect in control strips (Fig. 3C) but this treatment induced an
inhibition of EFS-elicited contractile responses in cholecystitic strips (56 % of inhibition; Fig. 3F).

**Effects of melatonin on neuromuscular function in acute cholecystitis**

We have previously reported that melatonin treatment was able to restore gallbladder neuromuscular function in aging (Gomez-Pinilla et al., 2006a). To determine whether this hormone had beneficial effects in the alterations described above, we treated the animals with 2.5 and 30 mg/Kg melatonin (MEL 2.5; MEL 30) as described in the Material and Methods section. Under these conditions, none of the melatonin doses used enhanced the amplitude of the contractile responses evoked by EFS (Fig. 4A), but the contractions partially recovered the sensitivity to TTX (85 and 77 % of inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 4B). Although the treatment dose-dependently increased the inhibitory effects of atropine and decreased the inhibitory effects of capsaicin significantly at some frequencies (Fig 4C & E), these changes were small. However, melatonin was able to protect nitrergic nerves because when L-NAME was added to the organ bath, the EFS-evoked contractile responses were enhanced in similar proportions to those found in control strips (91 and 85 % of enhancement for MEL 2.5 and MEL 30, respectively, at 5 Hz; Fig. 4D). These results indicate that melatonin had some effects on inflammation of the gallbladder, but there are some contractile disabilities related to the experimental method used in this study, as indicated in Material and Methods section, that melatonin treatment cannot restore.

Thus, we tested melatonin effects in animals that underwent the de-ligation protocol. When de-ligation was performed in animals that were not treated with melatonin, the neuromuscular function worsened, as indicated by the reduction in the EFS-induced contraction (Fig 5A & B). Taking into account the small amplitude of these contractions we did not apply antagonists/inhibitors of the neurotransmitters to determine the nature
of this response. However, when the animals were treated with melatonin 10 days before performing the surgical procedures and until the animal was sacrificed, there was a very noticeable improvement in gallbladder neuromuscular function. As shown in figure 5C, melatonin treatment increased the gallbladder neurogenic responses in a dose-dependent way. In the strips from animals treated with melatonin, the EFS-elicited responses recovered the sensitziveness to TTX (70 and 73 % of inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 6A) and atropine (73 and 76 % of inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz; Fig. 6B) to a level comparable with that seen in control tissue. Although capsaicin still induced a small inhibition of EFS-induced responses (5 and 10 % of inhibition for MEL 2.5 and MEL 30, respectively, at 25 Hz %; Fig. 6D), the reduction was significantly smaller than that found in inflamed tissue and at the highest frequencies this effect was not different from that registered in control tissue. In this experimental group, 30 mg/Kg melatonin also re-established the sensitivity to L-NAME (Fig. 6C) but this was not the case for 2.5 mg/Kg melatonin, suggesting that the effects of CBDL in the nitrergic function are exacerbated by the de-ligation procedure.

These results suggest that melatonin has prophylactic effects preventing neuromuscular damage during cholecystitis. To check whether melatonin also has a therapeutic role in the management of acute cholecystitis, melatonin treatment (30 mg/kg) was initiated after the onset of gallbladder inflammation. As represented in Figs 5C and 6, melatonin recovered gallbladder contractility in response to EFS and the pharmacological profile of the neurotransmission, indicating that the indoleamine can ameliorate the neuromuscular damage induced by acute cholecystitis.
These effects of melatonin could be related to its antioxidant and scavenger properties as indicated by the reduction in the lipidic peroxidation and the increase in the levels of GSH induced by melatonin treatment (Table 1).

**Effects of tempol on neuromuscular function in acute cholecystitis**

To test whether other antioxidants/scavengers have also beneficial effects on the impaired neuromuscular transmission in acute cholecystitis, we also tested the effects of tempol, a membrane permeable superoxide dismutase mimetic (Krishna et al., 1996). Administration of tempol in the drinking water for 14 days to guinea pigs that underwent the protocol of de-ligation prevented the functional impairment of EFS-induced contraction although to a lesser extent than 30 mg/kg melatonin (63 % and 26 % of recovery for MEL 30 and tempol, respectively, Fig 7A and B, *P* < 0.01). The recovery was accompanied by the normalization of the neurotransmission, since TTX, atropine, L-NAME and capsaicin had similar effects in tempol treated animals than in control ones (Fig. 7C, D,E and F). These prophylactic effects also correlated with a decrease in the MDA levels and an increase in the GSH content that were altered by the AC and DL protocols (Table 1).

**Discussion**

The current report shows that the impairment in guinea pig gallbladder neurotransmission evoked by inflammation was associated with a decrease in the contribution of the efferent plexus and the up-regulation of sensory afferent fibers. In addition, melatonin treatment caused an improvement in the neurogenic contractile response and the normalization of the different neural components that were probably related to its antioxidant and scavenger properties.

Our results indicate that EFS evokes a gallbladder response by the release of different inhibitory and excitatory neurotransmitters. The smaller contractile responses
to EFS in cholecystitic strips suggests the existence of an inflammation-induced impairment in the gallbladder intrinsic nerves, in agreement with previous results in human and animal models (McKirdy et al., 1994a; Parkman et al., 2000). However, the reduced gallbladder smooth muscle contractility to ACh found in cholecystitis (Gomez-Pinilla et al., 2006b; Parkman et al., 2000; Parkman et al., 1999a) could also contribute to the impaired neuromuscular function in inflamed tissue. The most striking finding in our study was the lack of sensitivity shown by cholecystitic strips to TTX, which could be explained by a direct release of neurotransmitter from nervous terminal. This was confirmed by the sensitivity of the EFS-induced responses to ω-conotoxin GVIA, a blocker of N type calcium channel located in the presynaptic membrane whose activation is necessary for neurotransmitter release. These results suggest that inflammation evokes a functional denervation in the gallbladder that avoids the genesis or propagation of action potential through efferent fibres. Alterations in the properties and/or expression levels of voltage-dependent Na⁺ channels have been implicated in a variety of pathological states, including inflammation of the viscera (Beyak et al., 2004; Stewart et al., 2003; Yoshimura et al., 2001). In this regard, the more common effect of inflammation on Na⁺ channels is the up-regulation of TTX-resistant slow (Nav1.8) (Beyak et al., 2004; Yoshimura et al., 2001). Alterations in the pharmacological profile of Na⁺ channels could also explain the TTX-resistant contractions reported in this study.

Classically, ACh released in response to EFS is the mainly excitatory component of the gallbladder contraction (Parkman et al., 1997; Yau and Youther, 1984). Here, we show that in control conditions atropine abolished EFS-induced contraction, while in inflamed tissue it just reduced EFS-elicited contraction about 50%, indicative of a functional denervation of the cholinergic component, similar to results previously described in inflamed gallbladder (Parkman et al., 2000).
Non adrenergic non cholinergic (NANC) neurotransmission in guinea pig gallbladder was described more than a decade ago (Mourelle et al., 1993) and NO is the main NANC neurotransmitter involved (Alcon et al., 2001; McKirdy et al., 1994b). Inflammation evokes a functional impairment in gallbladder nitrergic innervation as demonstrated by the lack of effects of L-NAME in cholecystic strips compared to control tissue. This result does not support the study from Parkman et al. (Parkman et al., 2000), where L-NAME only had effect in inflamed tissue indicating that normal gallbladder does not release NO from the intrinsic plexus. This is in conflict with the presence of nitrergic nerves described in guinea pig gallbladder (Mawe et al., 2006) and the functional data reported above.

Neurotransmitters released from sensory nerves evoked contraction or relaxation of the gallbladder (Maggi et al., 1989). In our study, sensory denervation with capsaicin had not effects in control conditions while it reduced EFS-elicited contractile response in inflammation, suggesting excitatory neurotransmitter release from sensory nerves in inflamed gallbladder. The major participation of the sensory innervation is a common finding in neurogenic inflammation (Sann et al., 1996). In the gallbladder, we have shown that aging, which is also related to increased oxidative stress, is associated to over-reactivity of sensory fibers (Gomez-Pinilla et al., 2006a).

The most important finding of our study is that melatonin has prophylactic and therapeutic effects on inflammation-induced impairment in gallbladder neuromuscular function. Thus, with 14 day melatonin treatment the EFS-induced contractile response recovered the sensitiveness to TTX, indicating that melatonin protects the voltage-dependent Na⁺-channels involved in the neural transmission of the action potential. Furthermore, the nitrergic innervation recovered its functionality and sensory fibers became less sensitive to EFS. However, melatonin itself did not improve the contractile
response to EFS unless the obstruction of the bile duct was relieved. Under these conditions, melatonin reversed the impairment in contractility in a dose-dependent manner, and fully recovered the different neural components stimulated by EFS. It must be pointed out that 2.5 mg/Kg melatonin had no effects on the nitrergic innervation after de-ligation, although this treatment was efficacious increasing the participation of these inhibitory nerves with the bile duct ligated. De-ligation itself worsened gallbladder contractility, as consequence probably of an increase in oxidative stress insult due to reperfusion of the organ once the mechanical stretch was alleviated. This is supported by the increase in the MDA levels indicative of lipidic peroxidation and oxidative stress injury found after de-ligation. On this basis, it appears that nitrergic innervation is especially sensitive to the enhanced oxidative stress after de-ligation. In agreement with this, we have recently reported a minor participation of nitrergic nerves in neuromuscular transmission in aging and its recovery after melatonin treatment (Gomez-Pinilla et al., 2006a). Furthermore, melatonin has been shown to have neurally-mediated actions in the gut regulating either cholinergic, nitrergic and/or sensory innervation (Reiter et al., 2003; Barajas-Lopez et al., 1996).

According to our results, melatonin not only protects against inflammation but also resolves the inflammation-induced impairment of neuromuscular function. Thus, when melatonin treatment started after the onset of gallbladder inflammation there was an enhancement of the contractile response to EFS that also recovered the neurotransmission pattern. However, the prophylactic administration of melatonin was more effective than the therapeutic one, which could be related to the increase in the antioxidant defences induced by the administration of melatonin prior to the oxidative insult.
It is well accepted that melatonin administration at pharmacological doses decreases free radical formation and leads to a substantial recovery of the major antioxidant enzymes (Reiter, 1998). Recent evidence has shown that melatonin has protective effects on liver and hepatic injury after extrahepatic bile duct ligation in rats (Ohta et al., 2005; Shiesh et al., 2000; Esrefoglu et al., 2005). Additional to liver and hepatic damage, free radical accumulation associated with bile duct ligation has been implicated in the genesis of gallstone (Eder et al., 1996). In this regard antioxidant treatment with melatonin not only reversed the increased oxidative stress, but also prevented gallstone formation (Shiesh et al., 2000). In our preparation, either prophylactic or therapeutic melatonin treatments were effective in reducing MDA levels and increasing the endogenous antioxidant defence GSH, indicating that melatonin antioxidant effects can be responsible for the improvement in the neuromuscular function. In fact, the treatment of the animals with the membrane permeant superoxide dismutase mimetic, tempol, also induced a significant improvement in the neuromuscular function of inflamed gallbladder, which is in agreement with other reports showing that tempol reduces the dysfunctions associated to oxidative stress insult (Chatterjee et al., 2000; Mehta et al., 2004). Collectively, our data suggest a prophylactic and therapeutic role of melatonin in experimental acute cholecystitis, a remarkable finding due to the lack of an effective pharmacological treatment for acute cholecystitis.

In conclusion, the results obtained in the present study indicate that inflammation impairs gallbladder neuromuscular function as the result of changes in the neural inputs to smooth muscle. These changes can be summarized as a denervation of efferent nerves together with a hyperactivity of afferent fibers. Melatonin significantly ameliorated the inflammation-related changes in gallbladder neuromuscular function.
transmission indicating its potential to combat inflammation-induced gallbladder damage.

Acknowledgements

The authors thank Rosario Moreno for technical assistance. Pedro J Gómez-Pinilla is recipient of a doctoral fellowship from Junta de Extremadura.
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Mehta SH, Webb RC, Ergul A, Tawfik A, Dorrance AM, Tawak A.


Footnotes:

This work was supported by Ministerio de Educacion y Ciencia (BFU 2004-0637) and Junta de Extremadura (2PR03A020).
Legends for figures

**Fig. 1.** Inflammation impairs EFS-elicited contractile responses in guinea pig gallbladder. A, original recordings showing guinea pig gallbladder contractile responses elicited by EFS (0.3 ms duration, 5-40 Hz, 350 mA, for 10 s every 3 min) applied to control and acute cholecistitic (AC) strips. Traces are typical of 26 to 18 strips for control and AC strips, respectively. B, superimposed recordings of the EFS-induced response to 25 Hz showing in more details the inflammation-related reduction in the peak amplitude and duration of the response. C and D, summary data of EFS induced-responses (peak amplitude in C and duration in D) in both experimental groups (* P < 0.01, ** P < 0.001, ANOVA).

**Fig. 2.** AC induces a TTX-resistant gallbladder response to EFS. Effects of 1 µM TTX on EFS-elicited contractile response in control (A) and AC gallbladder strips (B). After EFS was performed in control conditions (solid lines) strips were incubated for 20 min with the antagonist and EFS was repeated (dotted line). Note the lack of effects of TTX on inflamed tissue and the reduction in the response after incubation with 0.1 µM ω-conotoxin. Data are mean ± SEM. n = 7 and 6 strips for control and AC strips, respectively. (**) P < 0.01, ANOVA).

**Fig. 3.** AC impairs the efferent innervation and increases the excitability of sensory contractile fibers. Effects of 1 µM atropine, 100 µM of L-NAME and 10 µM of capsaicin on EFS-elicited contractile response in control (A, B, C) and AC gallbladder strips (D, E, F). After EFS was performed under control conditions (solid lines) strips were incubated for 20 min with the antagonist/inhibitor and EFS was repeated (dotted line). Note the lack of effects of L-NAME on inflamed tissue and the reduction in the
response after incubation with capsaicin. Data are mean ± SEM. n = 8-21 strips (* P < 0.05, ** P < 0.01, ANOVA).

**Fig. 4.** Melatonin treatment protects intrinsic neurons but does not improve EFS-induced contraction. (A) Effects of melatonin treatment (2.5 and 30 mg/kg) on EFS-induced gallbladder contractions in acute cholecystitis. Histograms represent the effects of 1 µM TTX (B), 1 µM atropine (C), 100 µM of L-NAME (D) and 10 µM of capsaicin (E) on EFS-elicited contractile response in control, AC and AC melatonin-treated gallbladder strips. Data are mean ± SEM. n = 5-18 strips. Note that EFS-induced responses recover TTX and L-NAME sensitivity, whereas melatonin has less effect on cholinergic and sensory fibers. (* P < 0.01 AC vs control † P < 0.05 MEL30 vs AC, δ P < 0.01 MEL 30 vs AC, ANOVA)

**Fig. 5.** Melatonin treatment improves the neurogenic damage exacerbated by the de-ligation procedure. A, original recordings showing guinea pig gallbladder contractile responses elicited by EFS (0.3 ms duration, 5-40 Hz, 350 mA, for 10 s every 3 min) applied to acute cholecystitic (AC) and de-ligated (DL) strips. Traces are typical of 16 and 17 strips for AC and DL strips, respectively. B, summary data of EFS induced-responses (peak amplitude) in both experimental groups (** P < 0.001, ANOVA). C, effects of melatonin treatment (2.5 and 30 mg/kg, DL + MEL 2.5 and DL + MEL 30, respectively; 30 mg/kg for four days after the onset of AC, DL + MEL 4 days) on EFS-elicited contractile response gallbladder strips from animals that underwent the de-ligation procedure. Data are mean ± SEM. (n = 12-28 strips, * P < 0.05 , ** P < 0.01, *** P < 0.001 vs AC, ANOVA).

**Fig. 6.** Melatonin treatment normalizes the different neural components stimulated by EFS. Effects of 100 µM TTX (A), 1 µM atropine (B), 100 µM of L-NAME (C) and 10 µM of capsaicin (D) on EFS-elicited contractile responses in control, AC and melatonin
treated gallbladder strips. Melatonin was administered to animals that underwent the de-ligation protocol (2.5 and 30 mg/kg, DL + MEL 2.5 and DL + MEL 30, respectively; 30 mg/kg for four days after the onset of AC, DL + MEL 4 days). After EFS was performed under control conditions strips were incubated for 20 min with the antagonist/inhibitor and EFS was repeated. Data are mean ± SEM. (n = 7-9 strips, * P < 0.01 vs control, δ P < 0.01 vs AC, ANOVA).

Fig. 7. Tempol treatment improves the neuromuscular damage exacerbated by the de-ligation procedure and normalizes the contribution of the different neural components in the inflamed gallbladder. A, original recordings showing guinea pig gallbladder contractile responses elicited by EFS (0.3 ms duration, 5-40 Hz, 350 mA, for 10 s every 3 min) applied to de-ligated strips (DL) from animals treated with 1mM Tempol (DL + Tempol 1 mM). Traces are typical of 17 and 20 strips for DL and DL + Tempol 1 mM, respectively. B, summary data of EFS induced-responses (peak amplitude) in those experimental groups (**P < 0.001 and *P < 0.01 vs DL, ANOVA). C, D, E and F, histograms showing the effects of 100 µM TTX, 1 µM atropine, 100 µM of L-NAME and 10 µM of capsaicin on EFS-elicited contractile responses in control, AC and DL + Tempol groups. Data are mean ± SEM. (n = 10-21 strips, *P < 0.01 vs control, δ P < 0.01 vs AC, ANOVA).
### Table 1.- Effect of acute cholecystitis, melatonin and tempol treatment on oxidative stress markers

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<td><strong>GSH level</strong></td>
<td>0.31 ± 0.05</td>
<td>0.35 ± 0.05</td>
<td>0.76 ± 0.07* δ</td>
<td>0.91 ± 0.06* δ</td>
<td>0.89 ± 0.06* δ</td>
<td>0.96 ± 0.04* δ</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM of fold increase respect to levels found in control tissue. N = 5-6 animals.

*P < 0.05, **P < 0.01 vs AC; δP < 0.05; δδP < 0.01 vs DL.
Figure 1

A  Control

1 mN/mg

5 Hz  10 Hz  15 Hz  25 Hz  40 Hz

3 min

AC

B

25 Hz

1 mN/mg

50 s

DC

C

D

Frequency (Hz)

Tension (mN/mg)

Control

AC

Frequency (Hz)

Time (s)

Control

AC

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Figure 2

A

Control

- ■ Control
- ▲ TTX

Tension (mN/mg)

Frequency (Hz)

B

Acute Cholecystitis

- ■ Control
- ▲ TTX
- ▼ ω-Conotoxin

Tension (mN/mg)

Frequency (Hz)
Figure 3

A. Control

B. Acute Cholecystitis

C. Control

D. Acute Cholecystitis

E. Control

F. Control
Figure 5

A

AC

DL

0.5 mN/mg

100 sec

B

C

Control

AC

DL + MEL 2.5

DL + MEL 30

DL + MEL 4 days

**

***

*
Figure 6

A  TTX

B  Atropine

C  L-NAME

D  Capsaicin

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>AC</th>
<th>DL+ MEL 2.5</th>
<th>DL + MEL 30</th>
<th>DL+ MEL 4 days</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inhibition (%)</strong></td>
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<tr>
<td>5 Hz</td>
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<td>10 Hz</td>
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<td>15 Hz</td>
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<td>25 Hz</td>
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<tr>
<td>40 Hz</td>
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</tbody>
</table>

**Increase (%)**

<table>
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<th>DL+ MEL 2.5</th>
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<tr>
<td>5 Hz</td>
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<td>25 Hz</td>
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<td>40 Hz</td>
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</tbody>
</table>
Figure 7

A. DL

DL

DL + Tempol 1 mM

0.5 mN/mg

3 min

B. Control

DL

DL + Tempol (1 mM)

Tension (mN/mg)

Frequency (Hz)

C. TTX

Inhibition (%)

Frequency (Hz)

D. Atropine

Inhibition (%)

Frequency (Hz)

E. L-NAME

Increase (%)

Frequency (Hz)

F. Capsaicin

Inhibition (%)

Frequency (Hz)

Control  AC  DL + Tempol (1 mM)