

**Neutrophil Recruitment and Articular Hyperalgesia in Antigen-
Induced Arthritis is Modulated by the Cholinergic Anti-
Inflammatory Pathway**

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Abstract

The cholinergic anti-inflammatory pathway (CAP) is a complex neuroimmune mechanism triggered by the central nervous system to regulate peripheral inflammatory responses. Understanding the role of CAP in the pathogenesis of rheumatoid arthritis (RA) could help develop new therapeutic strategies for this disease. Therefore, we investigated the participation of this neuro-immune pathway on the progression of experimental arthritis. Using an antigen-induced arthritis (AIA) model, we investigated in mice the effects of vagotomy or the pharmacological treatments with hexamethonium (peripheral nicotinic receptor antagonist), methyl-atropine (peripheral muscarinic receptor antagonist) or neostigmine (peripheral acetylcholinesterase inhibitor) on AIA progression. Unilateral cervical vagotomy was performed one week before the immunization protocol with methylated bovine serum albumin (mBSA), while the drugs administration was conducted during the period of immunization. On day twenty-one, six hours after the challenge with mBSA injection in the femur-tibial joint, the local neutrophil migration and articular mechanical hyperalgesia were assessed. Herein, we observed that vagotomy or blockade of peripheral nicotinic (but not muscarinic) receptors exacerbated the clinical parameters of this disease. Moreover, peripheral acetylcholinesterase inhibition by neostigmine-treatment promoted a reduction of neutrophil recruitment in the knee joint and articular hyperalgesia. Our results demonstrated that peripheral activation of CAP modulates experimental arthritis, providing a preclinical evidence of a potential therapeutic strategy for RA.

Key words: cholinergic anti-inflammatory pathway, vagus nerve, rheumatoid arthritis, acetylcholinesterase inhibitor, nicotinic receptor.

The central nervous system signals to the periphery through efferent neuronal pathways to orchestrate several steps of immune responses [1]. Moreover, several neuro-immune pathways, including those involving the autonomic nervous system, modulate inflammatory processes [2]. Among these pathways, the cholinergic anti-inflammatory pathway (CAP) has received significant attention, since parasympathetic integrity has been shown to be essential to maintain neurophysiological homeostasis and to control the inflammatory response [3,4]. In fact, electrical stimulation of the vagus nerve, the main component of the parasympathetic system, culminates in the release of T cell-derived acetylcholine, which binds in $\alpha 7$ nicotinic receptors expressed on splenic macrophages inhibiting pro-inflammatory cytokine production in endotoxemic mice [5].

Rheumatoid arthritis (RA) is a chronic autoimmune disease of unknown etiology that involves hyperplasia of synovial tissues and structural damage to cartilage and bone [6]. Several studies of RA have demonstrated that CAP manipulation can modulate arthritis development [7]. Indeed, electrical stimulation of the vagus nerve or nicotine administration improved clinical parameters and progression of the disease in experimental models, such as collagen-induced arthritis and adjuvant-induced arthritis [8-11]. However, many aspects of the mechanisms mediating these anti-inflammatory effects remain unclear.

The antigen-induced arthritis (AIA) is a useful and well-established experimental model to investigate the efficacy and safety of potential therapeutic agents and immune components involved in the RA progression, since arthritic mice and human RA share similar histopathological and immune features [12]. Thus, in the present study, we evaluated whether CAP manipulation during the period of immunization can affect the knee joint neutrophil migration and articular hyperalgesia in AIA model. We found that this neuroimmune pathway modulated the progression of arthritis by a mechanism dependent on the vagal signaling and the presence of peripheral nicotinic receptors. Moreover, neostigmine ameliorated the clinical

symptoms of experimental arthritis, suggesting that peripheral acetylcholinesterase modulation could be a coherent target to develop novel therapeutic strategies for RA.

Material and methods

Animals

Male BALB/c mice weighing 18–22g were housed in temperature-controlled rooms (22–25°C) in the animal facility of the Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil, and received water and food ad libitum. The study protocols followed the ethical guidelines of the Ribeirão Preto Medical School, University of São Paulo (São Paulo, Brazil).

Antigen-induced arthritis (AIA)

Mice were immunized with methylated bovine serum albumin (mBSA, Sigma-Aldrich, St. Louis, MO, USA) as described previously [13]. Briefly, mice were immunized with subcutaneous injections of an emulsion containing mBSA (500 µg, Sigma-Aldrich, St. Louis, MO, USA) and Freund's complete adjuvant (CFA, 1 mg.ml⁻¹ of inactivated *Mycobacterium tuberculosis*, Sigma-Aldrich, St. Louis, MO, USA). A booster injection of mBSA, again dissolved in CFA, was given on day seven after the first immunization. On day 21, mice were challenged with a femur-tibial injection of 10 µg of mBSA dissolved in PBS.

Determination of joint neutrophil infiltration

Neutrophil infiltration in the knee joints was assessed 6 h after intra-articular challenges with mBSA by counting the number of cells harvested from articular cavities as previously described [14]. Briefly, the articular infiltration of neutrophils was assessed in mice euthanized in CO₂ chamber by washing the femur-tibial joint three times with 3.3 µl of

PBS + EDTA (0.2 mol/L) after which the number of neutrophil cells were counted in a Neubauer chamber. In all experiments, just one person carried out blindly the neutrophil counts.

Articular hypernociception evaluation

The articular hypernociception of the femur-tibial joint was evaluated as previously described [13]. A non-nociceptive tip probe with an area size of 4.15 mm² was used. An increasing perpendicular force was applied to the central area of the hind paw to induce flexion of the femur-tibial joint, followed by paw withdrawn. The electronic pressure-meter apparatus automatically recorded the intensity of the force applied when the paw was withdrawn. The test was repeated for three subsequently consistent measurements (i.e. the variation among these measurements was less than 1 g). The results were expressed as the flexion elicited withdrawal threshold in grams (g). In all experiments, just one person carried out blindly the evaluation of articular hypernociception.

Experimental protocols

All schematic protocols are shown in Figure 1. In the first experiment, we subjected mice to unilateral right cervical vagotomy or sham surgery (control group) one week before the beginning of the immunization protocol. As described by previous studies, unilateral left and right cervical vagotomy influenced immune response in a similar way [15]. In brief, mice were previously anesthetized with ketamine and xylazine and a ventral cervical midline incision was performed to expose the right cervical vagus trunk, which was transected. In sham-operated mice, the vagus nerve was exposed but was not transected.

In a second experimental protocol, the roles of peripheral nicotinic and muscarinic receptors were investigated on the development of AIA using hexamethonium and methyl-atropine, nicotinic and muscarinic receptor antagonists that do not cross the blood-brain barrier (BBB), respectively. In this experiment, mice were treated twice a day with methyl-atropine (3 and 10 mg/kg; s.c.), hexamethonium (3 and 10 mg/kg; s.c.) or saline (vehicle) from days 4 to 10 after the first immunization (Fig. 1).

Subsequently, we performed a third experiment investigating the effect of CAP activation on the AIA progression. Here the role of neostigmine, a parasympathomimetic drug that inhibits acetylcholinesterase and does not cross BBB, were assessed on AIA development. In this experiment, mice were treated twice a day with neostigmine (12.5, 25 and 50 µg/kg; s.c.) or saline (vehicle) from days 4 to 10 after the first immunization (Fig. 1).

Unilateral cervical vagotomized animal showed a temporary weight loss as compared with the sham-vagotomized group, but recovered the original body weight 3 days after the surgical procedure. No mice showed significant side effect (behavioral changes, weight loss) during the chronic pharmacological treatment with methyl-atropine (Sigma-Aldrich, St. Louis, MO, USA, catalog number M13000000), hexamethonium (Sigma-Aldrich, St. Louis, MO, USA, catalog number H0879) or neostigmine (Sigma-Aldrich, St. Louis, MO, USA, catalog number N2001).

Statistical analyses

Data are expressed as the mean \pm SEM of 5-7 animals per group. Statistical analysis was performed by One-way Analysis of Variance (One-way ANOVA) combined with the Bonferroni's Multiple Comparison Test using GraphPad Prism Software (version 5.01 for Windows, GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered significant.

Results

In the vagotomy experiment, where part of the mice underwent an early unilateral cervical **vagus nerve transection** (Fig. 1), we found that the neutrophil recruitment to knee joint and articular hyperalgesia had significantly increased 6 h after mBSA challenge in the vagotomized mice when compared with sham-vagotomized animals (Fig. 2A and 2B, respectively).

Aiming to identify the type of peripheral acetylcholine receptor that mediates immunomodulatory properties associated with the parasympathetic system, we investigated the effect of hexamethonium or methyl-atropine treatment on AIA (Fig. 1). Similarly to vagotomy, the treatment with hexamethonium, but not with methyl-atropine, exacerbated neutrophil migration and articular hyperalgesia (Fig. 3A and 3B, respectively), indicating that peripheral nicotinic receptors are key mediators of the modulation of the adaptive immune response during the period of immunization (generation phase of the disease).

Finally, since interruption or blocked on transmission of the parasympathetic system modulates inflammation, we investigated the possible anti-inflammatory effects of neostigmine, a parasympathomimetic drug that inhibits peripheral acetylcholinesterase, on AIA development. In this experiment, we observed that the treatment during the period of immunization with neostigmine (Fig. 1) prevented significantly the knee joint neutrophil recruitment and articular hyperalgesia in a dose dependent-manner (Fig. 4A and B, respectively).

Discussion

Neutrophils are the most abundant cells found in the synovial fluid during the acute phases of arthritis development in AIA and RA [16]. Furthermore, our group have

demonstrated that excessive neutrophil trafficking toward the knee joint is crucial to trigger hyperalgesic behaviors [17]. In fact, experimental studies have also shown that the reduction of neutrophil recruitment attenuates arthritis progression and severity [18].

Based on the experimental evidences that CAP regulates immune responses in experimental models of RA [8-11] and clinical observations that observed autonomic dysfunction in autoimmune diseases [19], it is reasonable to claim that CAP manipulation could be strategically useful for RA treatment. In this work we demonstrated that the interruption of parasympathetic signals by vagotomy surgery enhances the neutrophil migration and hyperalgesia in AIA. Consistently, others studies have demonstrated that vagotomy worsened clinical signals in other experimental models of arthritis, particularly in collagen-induced arthritis [8,9,11].

Additionally, previous studies have demonstrated that nicotinic receptors handle the propagation of the CAP signaling [3]. However, the participation of the muscarinic receptors has not been discarded in the modulation of arthritis development; on the contrary, it was clearly demonstrated that these receptors mediate anti-inflammatory properties as well [20]. In the AIA model, peripheral nicotinic (but not muscarinic) receptors were associated with arthritis development, as only hexamethonium administration during the induction of the adaptive immune response enhanced the inflammatory parameters.

Despite others reports that have suggested nicotine for RA treatment [8,9,11], a possible implementation of nicotine in the therapeutic schedule is still under debate due to its pro-inflammatory side-effects in experimental arthritis when administrated for prolonged periods [21]. Therefore, others compounds that activate CAP, such as parasympathomimetic agents, could be an alternative to nicotine administration. Intrathecal neostigmine administration has been described to induce analgesia in the inflamed joints of rats [22], but there are no studies demonstrating the effect of this drug on the immune response. In the

present study, neostigmine strongly reduced neutrophil migration and pain sensitivity in the AIA model. One point that need to be mentioned is that our result is not due to a direct effect of neostigmine on the neutrophil migration induced by knee joint mBSA challenge but by an effect exerted during the effector phase of immunization (participation of adaptive immune response) because this drug was injected only two weeks before the i.a. mBSA injection, suggesting that neostigmine has marked immunomodulatory properties. In fact, conventional drugs currently used for RA treatment approach these properties, such as methotrexate and leflunomide, and are able to modulate different subsets of leucocytes (Cronstein 2005; Breedveld et al., 2000). In accordance, a recent report demonstrated that galantamine, another acetylcholinesterase inhibitor, also showed anti-inflammatory effects on adjuvant-induced arthritis in rats [23].

The present study shows some limitations that have to be pointed out. First, recent studies has demonstrated a physiological integration between the parasympathetic and sympathetic nervous systems in the CAP [1]. For example, a previous study demonstrated the influence of sympathetic systems on the AIA progression [24]. Altogether, our and this study could evidence a real connection between these two systems in RA progression. In our experimental conditions, hexamethonium can act as antagonist at the nicotinic acetylcholine receptors located in both sympathetic and parasympathetic ganglia and, therefore, this drug could block the neural transmission of both autonomic systems. Second, vagus nerve is composed by efferent and afferent nerves [25] and does not innervate directly the knee joints [26], suggesting that others central and peripheral components (indirect manner), such as α_7 receptors activation, could be involved in the CAP functioning, as observed in the reduction of inflammation in skeletal muscle and skin (Leite et al., 2014; Kurzen et al., 2004). Future studies are needed to decode the CAP components involved in inflammation regulation.

In summary, our data showed for the first time the immunomodulatory properties of CAP in two inflammatory RA parameters not yet investigated in a well-established model of arthritis complementing previous studies [8-11] that demonstrated that this neuro-immune mechanism is an approach to be explored to control inappropriate and massive inflammatory response observed in RA.

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References

1. Olofsson PS, Rosas-Ballina M, Levine YA, Tracey KJ. Rethinking inflammation: neural circuits in the regulation of immunity. *Immunol Rev* 2012;**248**:188-204.
2. Ordovas-Montanes J, Rakoff-Nahoum S, Huang S, Rioll-Bianco L, Barreiro O, von Andrian UH. The Regulation of Immunological Processes by Peripheral Neurons in Homeostasis and Disease. *Trends Immunol* 2015;**36**:578-604.
3. Borovikova LV, Ivanova S, Zhang M, Yang H, Botchkina GI, Watkins LR, et al. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature* 2000;**405**:458-62.
4. Tracey KJ. The inflammatory reflex. *Nature* 2002;**420**:853-59.

- 240 5. Rosas-Ballina M, Olofsson PS, Ochani M, Valdés-Ferrer SI, Levine YA, Reardon C, et al.
 241 Acetylcholine-synthesizing T cells relay neural signals in a vagus nerve circuit. *Science*
 242 2011;**334**:98-101.
- 243 6. Firestein GS. Evolving concepts of rheumatoid arthritis. *Nature* 2003;**423**:356-61.
- 244 7. van Maanen MA, Vervoordeldonk MJ, Tak PP. The cholinergic anti-inflammatory
 245 pathway: towards innovative treatment of rheumatoid arthritis. *Nat Rev Rheumatol*
 246 2009;**5**:229-32.
- 247 8. van Maanen MA, Lebre MC, van der Poll T, LaRosa GJ, Elbaum D, Vervoordeldonk MJ,
 248 et al. Stimulation of nicotinic acetylcholine receptors attenuates collagen-induced arthritis
 249 in mice. *Arthritis Rheum* 2009;**60**:114-22.
- 250 9. Li T, Zuo X, Zhou Y, Wang Y, Zhuang H, Zhang L, Zhang H, et al. The vagus nerve and
 251 nicotinic receptors involve inhibition of HMGB1 release and early pro-inflammatory
 252 cytokines function in collagen-induced arthritis. *J Clin Immunol* 2010;**30**:213-20.
- 253 10. Levine YA, Koopman FA, Faltys M, Caravaca A, Bendele A, Zitnik R, et al.
 254 Neurostimulation of the cholinergic anti-inflammatory pathway ameliorates disease in rat
 255 collagen-induced arthritis. *PLoS One* 2014;**9**:e104530.
- 256 11. Wu S, Luo H, Xiao X, Zhang H, Li T, Zuo X. Attenuation of collagen induced arthritis
 257 via suppression on Th17 response by activating cholinergic anti-inflammatory pathway
 258 with nicotine. *Eur J Pharmacol* 2014;**735**:97-104.
- 259 12. Brackertz D, Mitchell GF, Vadas MA, Mackay IR, Miller JF. Studies on antigen-induced
 260 arthritis in mice. II. Immunologic correlates of arthritis susceptibility in mice. *J Immunol*
 261 1977;**118**:1639-44.
- 262 13. Pinto LG, Cunha TM, Vieira SM, Lemos HP, Verri WA Jr, Cunha FQ, et al. IL-17
 263 mediates articular hypernociception in antigen-induced arthritis in mice. *Pain*
 264 2010;**148**:247-56.

- 265 14. Grespan R, Fukada SY, Lemos HP, Vieira SM, Napimoga MH, Teixeira MM, et al.
 266 CXCR2-specific chemokines mediate leukotriene B4-dependent recruitment of neutrophils
 267 to inflamed joints in mice with antigen-induced arthritis. *Arthritis Rheum.* 2008;**58**:2030-
 268 40.
- 269 15. van Westerloo DJ, Giebelen IA, Florquin S, Daalhuisen J, Bruno MJ, de Vos AF, et al.
 270 The cholinergic anti-inflammatory pathway regulates the host response during septic
 271 peritonitis. *J Infect Dis* 2005 191;**12**:2138-48.
- 272 16. Cascão R, Rosário HS, Souto-Carneiro MM, Fonseca JE. Neutrophils in rheumatoid
 273 arthritis: More than simple final effectors. *Autoimmun Rev* 2010;**9**:531-5.
- 274 17. Cunha TM, Verri WA Jr. Neutrophils: are they hyperalgesic or anti-hyperalgesic? *J*
 275 *Leukoc Biol.* 2006;**80**:727-8.
- 276 18. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a
 277 murine model of rheumatoid arthritis. *J Immunol* 2001;**167**:1601-8.
- 278 19. Mravec B. Autonomic dysfunction in autoimmune diseases: consequence or cause? *Lupus*
 279 2007;**16**:767-8.
- 280 20. Reardon C, Duncan GS, Brüstle A, Brenner D, Tusche MW, Olofsson PS, et al.
 281 Lymphocyte-derived ACh regulates local innate but not adaptive immunity. *Proc Natl*
 282 *Acad Sci U S A.* 2013;**110**:1400-1405.
- 283 21. Yu H, Yang YH, Rajaiah R, Moudgil KD. Nicotine-induced differential modulation of
 284 autoimmune arthritis in the Lewis rat involves changes in interleukin-17 and anti-cyclic
 285 citrullinated peptide antibodies. *Arthritis Rheum* 2011;**63**:981-91.
- 286 22. Buerkle H, Boschin M, Marcus MA, Brodner G, Wüsten R, Van Aken H. Central and
 287 peripheral analgesia mediated by the acetylcholinesterase-inhibitor neostigmine in the rat
 288 inflamed knee joint model. *Anesth Analg* 1998;**86**:1027-32.

23. Gowayed MA, Refaat R, Ahmed WM, El-Abhar HS. Effect of galantamine on adjuvant-induced arthritis in rats. *Eur J Pharmacol* 2015;**764**:547-53.
24. Ebbinghaus M, Gajda M, Boettger MK, Schaible HG, Bräuer R. The anti-inflammatory effects of sympathectomy in murine antigen-induced arthritis are associated with a reduction of Th1 and Th17 responses: *Ann Rheum Dis* 2012;**71**:253-61.
25. Berthoud HR, Neuhuber WL. Functional and chemical anatomy of the afferent vagal system. *Auton Neurosci.* 2000;**85**:1-17.
26. Schaible HG, Straub RH. Function of the sympathetic supply in acute and chronic experimental joint inflammation. *Auton Neurosci.* 2014;**182**:55-64.

Legends

Figure 1. Schematic representation of the experimental protocols used in the study. Mice were immunized with an emulsion containing mBSA and Freund's complete adjuvant (CFA). Booster injection of mBSA dissolved in CFA was given on day 7. Mice were subjected to vagotomy one week before the beginning of immunization (day 0) or treated twice a day with methyl-atropine, hexamethonium, neostigmine or vehicle from day 4 to day 10. Mice were challenged with an i.a. injection of mBSA on day 21. Six hours after mBSA-challenge, neutrophil migration into the knee joint and articular mechanical hyperalgesia was assessed.

Figure 2. Vagotomy exacerbates the clinical parameters in the AIA model. Mice were vagotomized seven days before first immunization. (A) Neutrophil migration and (B) mechanical hyperalgesia were evaluated 6 h after articular injection of saline or mBSA (10 ug/cavity). Values are represented as mean and SEM (n=5-7). n.d.= not detectable. * p<0.05 versus mBSA-immunized mice after mBSA challenge. Analysis of variance with Bonferroni test was applied.

Figure 3. Blockade of peripheral nicotinic, but not muscarinic, receptors increases neutrophil migration and mechanical hyperalgesia in AIA model. Prior to intra-articular (i.a.) challenge with mBSA (10 ug/cavity) on day 21, mBSA-immunized mice were treated with methyl-atropine (3 and 10 mg/kg; s.c.) or hexamethonium (3 and 10 mg/kg; s.c.) during the period from day 4 to day 10 after first immunization. (A) Neutrophil migration and (B) mechanical hyperalgesia were evaluated six h after articular injection of saline or mBSA. Values are represented as mean and SEM (n=5). n.d.= not detectable. * $p<0.05$ versus mBSA-immunized mice after mBSA challenge. Analysis of variance with Bonferroni test was applied.

Figure 4. Neostigmine treatment ameliorates clinical symptoms in the AIA model. Prior to intra-articular (i.a.) challenge with mBSA (10 ug/cavity) on day 21, mBSA-immunized mice were treated with neostigmine (12.5, 25 and 50 μ g/kg; s.c.) during the period from day 4 to day 10 after first immunization. (A) Neutrophil migration and (B) mechanical hyperalgesia were assessed six h after articular injection of saline or mBSA. Values are represented as mean and SEM (n=5). n.d.= not detectable. * $p<0.05$ versus mBSA-immunized mice after mBSA challenge. Analysis of variance with Bonferroni test was applied.

Figure 1

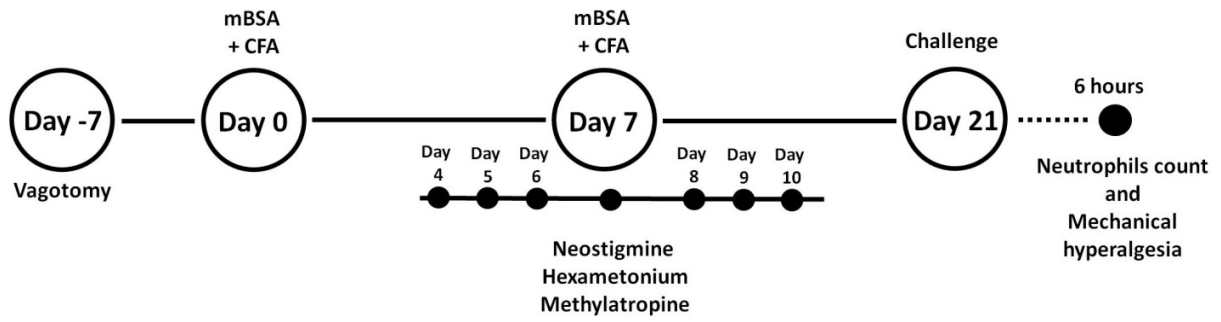


Figure 2

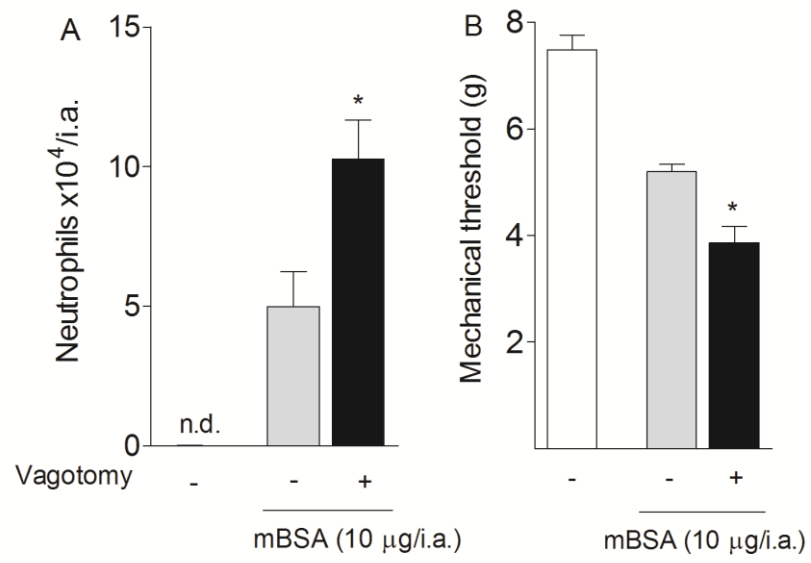
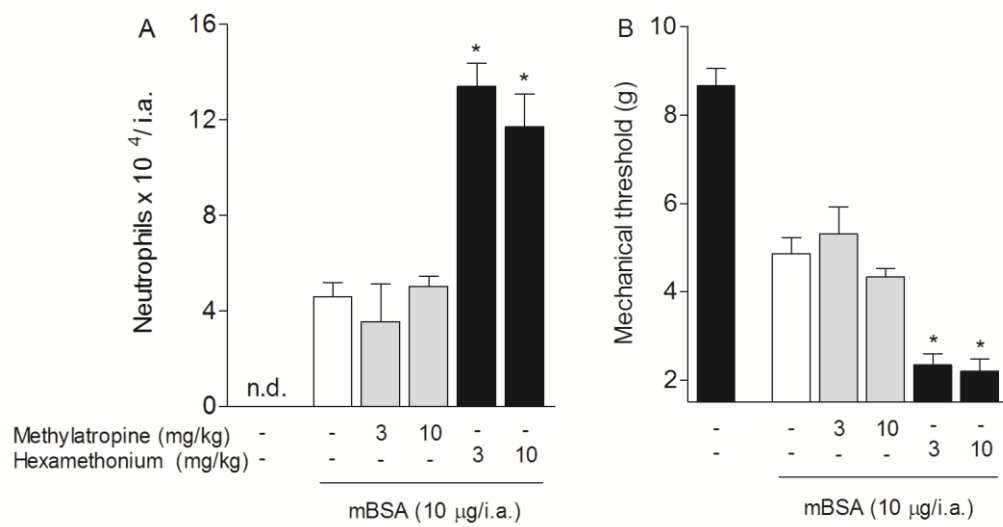
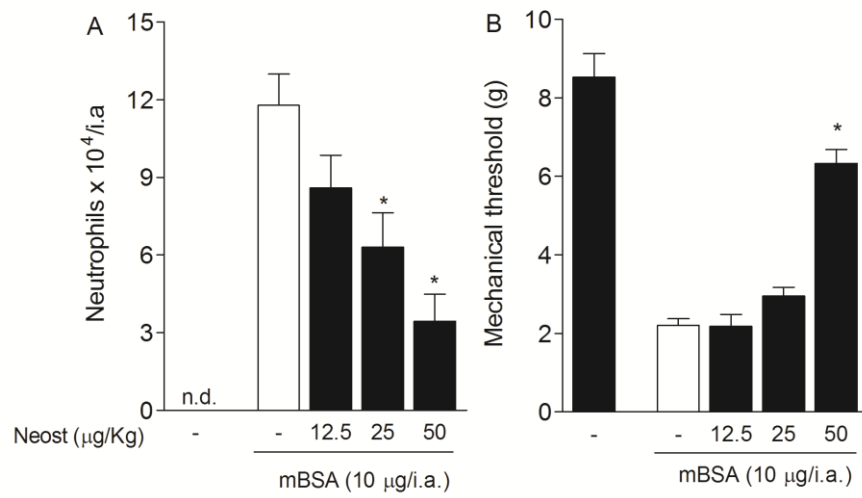


Figure 3



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Figure 4



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