Neuroinflammatory effects of tDCS in ovariectomized rats with chronic inflammation

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ABSTRACT

Introduction: Postmenopausal women are more susceptible to chronic conditions, such as osteoporosis, arthritis, and other inflammatory diseases. We investigated the effects of transcranial direct current stimulation (tDCS) on biomarker levels in ovariectomized rats subjected to an inflammatory model.

Methods: Twenty adult female Wistar rats underwent ovariectomy and complete Freund's adjuvant (CFA)-induced inflammation. We divided them into 2 groups: OAS (sham tDCS) and OAT (active tDCS). Fifteen days later, the rats underwent bimodal tDCS treatment (20 min, 0.5 mA, 8 days). After 24 h of the last tDCS session, we killed the rats and collected tissue samples (hypothalamus, cerebral cortex, and brainstem) for biomarker analysis by ELISA. We removed the paws for histological analysis.

Results: Active tDCS increased hypothalamic and cortical TNF-α and NGF levels, hypothalamic and brainstem IL-1β levels, and hypothalamic IL-10 levels. Histology of paws showed an inflammatory profile. We observed a small tDCS effect, not statistically significant.

Discussion: Bimodal tDCS had an effect on the central inflammatory axis, with a small effect on the peripheral site as evaluated by histology in the current study.

Keywords: *Inflammation; Cytokines; Estrogen; Ovariectomy; tDCS*

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INTRODUCTION

Menopause is the age-related loss of ovarian function and represents a state of profound estrogen deprivation[1]. It could be responsible, in part, for the elevation of pro-inflammatory cytokines seen in women of advanced age[2]. Postmenopausal women are more susceptible to chronic conditions, such as osteoporosis, arthritis, and other inflammatory diseases, which are associated with changes in the expression and secretion of pro-inflammatory cytokines[3]. Also, cognitive impairments are common during menopause, which is marked by a decrease in hormone levels[4].

Interestingly, estrogen deficiency may result in a significant increase in inflammatory factors in the blood, such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β)[5]. TNF-α levels increase abruptly after 51 years of age[6], which is the average age of menopause. Moreover, older postmenopausal women often present with suppressed nerve growth factor (NGF) expression[7] and significantly decreased serum IL-10 levels[8]. It is important to point out that they show greater upregulation of the genes involved in inflammation and immune function than older men[9,10].

Changes observed during menopause may also affect central nervous system structures related to hormones and inflammatory systems. The hypothalamus is a part of the brain that controls body temperature and may be involved in hot flashes (HFs) by increasing gonadotropin-releasing hormone secretion[11].

Also, increased brainstem activity has been detected before the detectable onset of HFs by using functional magnetic resonance imaging[12], and insular and prefrontal activity appeared following HF onset. The menopausal transition period has pronounced effects on the human brain structure, connectivity, and energy metabolism, which is related to an adaptive process serving the transition into later life.

Preclinical studies suggest that ovariectomy affects inflammatory functions under basal conditions, but little is known about the effects of ovariectomy on inflammatory responses induced under immune stress conditions[13]. In female rats, the potential effect of ovariectomy on inflammatory pain has not been elucidated. It is important to highlight that, in ovariectomized female rats, only high concentrations of formalin can demonstrate inflammatory and nociceptive responses[14-16].

Transcranial direct current stimulation (tDCS), a technique that non-focally modulates plastic changes induced by pain-related neural networks, can be a useful tool in menopause management. A previous study by our research group showed both immediate and long-lasting antinociceptive effects of repeated sessions of bicephalic tDCS in a rat model of chronic inflammation[17]. In another study by our group, ovariectomized rats showed a hypernociceptive response associated with changes in peripheral and central brain-derived neurotrophic factor (BDNF) levels; cathodal tDCS treatment partially reversed the mechanical hyperalgesia response and completely reversed the decreased cortical BDNF levels[18].

In this context, it is important to develop new studies on the effects of tDCS on inflammatory disease associated with low estrogen levels. Therefore, considering the need to develop novel strategies for menopause management, we investigated the effects of tDCS on central TNF-α, IL-1β, IL-10, and NGF levels in ovariectomized rats subjected to a model of chronic inflammation.

METHODS

Animals

We randomized 20 female Wistar rats (90 days old, 200–300 g) by weight and housed them in groups of 3 to 4 animals per polypropylene cage (49 cm × 34 cm × 16 cm) with sawdust-covered floor. We maintained all rats in a controlled environment (22 ± 2°C) under a standard light–dark cycle (lights-on at 7 a.m. and lights-off at 7 p.m.), with water and chow (Nuvital, Porto Alegre, RS, Brazil) *ad libitum*. The institutional Animal Care and Use Committee (GPPG-HCPA protocol no. 2014.0262) approved all experiments and procedures, which were performed in accordance with the Guide for the Care and Use

of Laboratory Animals, 8th ed. The experimental protocol complied with the ethical and methodological standards of the ARRIVE guidelines[19].

Experimental design

We allowed the rats to acclimate to the study environment for 14 days before the beginning of the experiment and, subsequently, randomized them by weight. On day 1, after performing ovariectomy with complete Freund's adjuvant (CFA)-induced inflammation via injection into the footpad under anesthesia, we divided the rats into the following 2 groups: OAS (treated with sham tDCS) and OAT (treated with active tDCS). Fifteen days later, the rats underwent daily tDCS sessions for 8 consecutive days. An experimenter blinded to group allocation performed biochemical and histological analyses.

Surgical procedures

We anesthetized the rats with 4% isoflurane for induction and 2% for maintenance and performed bilateral ovariectomy as described previously[20]. Surgery consisted of a dorsolateral transverse skin incision between the last rib and pelvis, followed by muscle dissection to expose the abdominal cavity. The ovary is in a fat pad beneath the muscles. We grasped the periovarian fat to lift and exteriorize the ovary. We crushed and ligated the fallopian tube, and then removed the ovary by cutting above the clamped area. We closed the muscles and skin incision with polyglactin and nylon sutures. We repeated the same procedure on the other side for bilateral ovariectomy. All rats received tramadol chlorhydrate (5 mg/kg, i.p.) for postoperative pain relief.

CFA-induced inflammation

We purchased CFA from Sigma Chemical Co. (St Louis, MO, USA). The protocol used for CFA-induced inflammation is similar to previously described protocols[21]. Briefly, we anesthetized the rats with isoflurane and induced inflammation via a single 100 µL intradermal injection of heat-killed *Mycobacterium tuberculosis* suspended in paraffin oil and mannide monooleate (1 mg/1 mL) into the right footpad.

Vaginal smear

Ten days after surgery, we obtained vaginal smears once daily to confirm hormonal status. We obtained and analyzed the samples as described previously[22].

Transcranial direct current stimulation (tDCS)

After 14 days of CFA administration, the rats underwent a 20 min session of bicephalic tDCS every morning for 8 days, as described previously[23,24].

Tissue collection

After 24h of the last tDCS session, we killed the rats by decapitation and collected brain tissue samples

(hypothalamus, cerebral cortex, and brainstem). We kept the samples frozen at −80°C until analysis.

Biochemical assays

We determined TNF-α, IL-1β, IL-10, and NGF levels by sandwich ELISA using monoclonal antibodies specific for each measurement (R&D Systems, Minneapolis, MN, USA). We measured total protein by Bradford's method using bovine serum albumin as standard and expressed the data as pg/mg of protein.

Histological scoring

For histological analysis, we excised the hind paws and fixed them in 10% buffered formalin for 7 days. We then decalcified the paws with 10% nitric acid for 27 h. We sectioned tissues and embedded them in paraffin. We prepared the slides by staining the tissue sections with hematoxylin and eosin. A pathologist blinded to treatment allocation performed a histological analysis of the tibiotarsal joint (ankle region). By optical microscopy, the pathologist graded the histological slides according to the percentage of infiltrating inflammatory cells compared with the surrounding tissue (0 = absent; 1 = mild [1%–10%]; 2 = moderate [11%–50%]; and 3 = severe [51%–100%]) and quantified the number of medium microscopic power fields (200×) occupied by the inflammatory process. We identified cells according to their morphological characteristics.

Statistical analysis

We expressed the data as mean and standard error of the mean (SEM). In biochemical assays, we used Student's t test for independent samples to compare the effects of tDCS. We analyzed the histological data using the Mann-Whitney test for independent samples. We considered differences to be statistically significant at $P < 0.05$. We used SPSS 20.0 for statistical analysis.

RESULTS

Assessment of hormonal status

The vaginal smears of the ovariectomized rats presented an acyclical pattern exhibiting only the metestrus and diestrus stages, thus confirming the animal model of menopause (n = 8–10 per group, data not shown).

Hypothalamic biomarker levels

Active tDCS induced an increase in hypothalamic TNF-α, IL-1β, IL-10, and NGF levels (Student's t test, $P < 0.01$ and $P < 0.001$ for IL-10 and NGF; Figure 1A).

Cerebral cortex biomarker levels

Active tDCS induced an increase in cerebral cortex TNF-α and NGF levels (Student's t test, $P < 0.05$ and $P < 0.02$, respectively; Figure 1B), with no difference between the groups in IL-1β or IL-10 levels (Student's t test, $P > 0.05$; Figure 1B).

Brainstem biomarker levels

Active tDCS induced an increase in brainstem IL-1β levels (Student's t test, $P < 0.03$; Figure 1C). There was no significant difference between the groups in TNF-α, IL-10, or NGF levels (Student's t test, $P > 0.05$; Figure 1C).
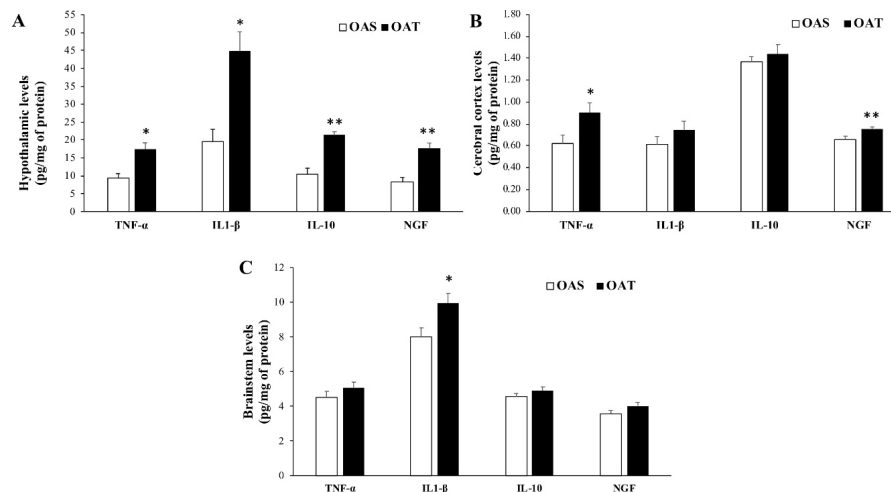

Figure 1: Biomarker levels in central nervous system (CNS) structures. Data are presented as mean ± standard error of the mean (SEM) of pg/mg of protein. OAS (ovariectomized + CFA + sham tDCS) and OAT (ovariectomized + CFA + tDCS) (n = 9 per group). A: Hypothalamic levels (Student's t test, $*P < 0.01$ and $**P < 0.001$); B: Cerebral cortex levels (Student's t test, $*P < 0.05$ and $**P < 0.02$); C: Brainstem levels (Student's t test, $*P < 0.03$).

Histological analysis

Figure 2 shows the histological findings for each group. We observed an inflammatory infiltrate of lymphocytes, plasmocytes, macrophages, and, more rarely, neutrophils in reaction to CFA injection in the subcutaneous tissues of the ankle joint. We also observed the formation of giant cells. The OAT group had lower inflammation scores, but there was no statistically significant difference in histological scoring between the groups (Mann-Whitney test, $P > 0.05$; Table 1).
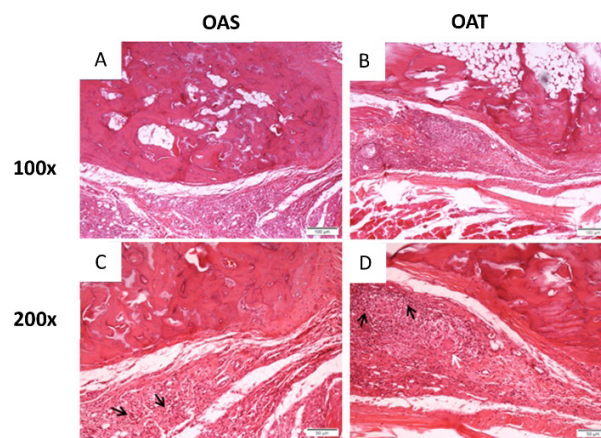

Figure 2: Hematoxylin and eosin-stained sections from the hind paws of rats (100× and 200× magnification) (n = 3-5 per group). A and C: OAS group showing inflammatory reaction with lymphocytes and plasma cells; B and D: OAT group showing inflammatory reaction with mononuclear cells (black arrows) and macrophage aggregates (white arrow). There was no significant difference between the groups; Mann-Whitney test, $P > 0.05$.

Table 1: Histological analysis of inflammatory processes in the tibiotarsal joint.

Groups	OAS		OAT		
	Median	(25%–75%)	Median	(25%–75%)	P*
Histological score	3.000	(0.5–4)	2.500	(0.25–4)	0.9993
Microscopic fields	22.00	(0.5–29.5)	15.00	(0–27)	0.8053

OAS: ovariectomized + CFA + sham tDCS; OAT: ovariectomized + CFA + tDCS.

* No significant difference (Mann-Whitney test).

DISCUSSION

In the present study, our data demonstrated that ovariectomized rats with chronic inflammation treated with tDCS had increased levels of all investigated biomarkers: TNF-α in the hypothalamus and cerebral cortex; IL-1β in the hypothalamus and brainstem; and IL-10 in the hypothalamus. In addition, tDCS induced an increase in NGF levels in the hypothalamus and cerebral cortex. It is important to note that tDCS-treated rats showed lower inflammation scores indicating a reduction in the number of inflammatory cells in the subcutaneous tissues of the ankle joint, although no statistical significance was reached.

Interestingly, a previous study highlighted that multiple sessions of tDCS triggered an inflammatory effect on the rat brain[25]. We showed that ovariectomy reduced hypothalamic and cerebral cortex BDNF levels, but tDCS reversed only the cerebral cortex levels[18]. Also, tDCS decreased hippocampal TNF levels in rats in a chronic pain model[26]. Moreover, bicephalic tDCS reduced IL-1β and IL-10 levels in the spinal cord and increased TNF-α levels in the cerebral cortex and spinal cord in rats subjected to neuropathic pain[24].

In the current study, we observed an increase in hypothalamic IL-10 levels in tDCS-treated rats. IL-10 is a cytokine released during the resolution phase of inflammation that prevents tissue damage caused by infection and inflammation[27,28]. Therefore, we hypothesized that tDCS potentiates an inflammatory state in ovariectomized rats with chronic inflammation by modulating the neuroinflammatory pathway.

In neuromodulator analysis, we observed an increase in NGF levels in the hypothalamus and cerebral cortex of rats treated with bimodal tDCS. NGF can elicit the release of inflammatory mediators, as well as NGF itself, resulting in a positive feedback loop[29,30]. It also sensitizes adjacent nociceptive neurons in response to inflammation[31]. In our previous study of ovariectomized rats, cathodal tDCS had neuromodulatory effects on mechanical hyperalgesia and on the cortical levels of BDNF[18], another neurotrophin involved in peripheral and central sensitization. It is noteworthy that tDCS is a neuromodulatory technique that induces potent neuroplastic changes, as observed in preclinical[32] and clinical studies[33]. Although its mechanism is not fully understood, our findings highlight an interaction of the immune system with the neural circuitry in the model of ovariectomized rats with chronic inflammation promoted by tDCS.

There is growing evidence of a relationship between tDCS neuromodulation and immune system activation, and the effects of this technique are neuronally and non-neuronally driven[34]. Despite the changes in central biomarkers triggered by tDCS, in the peripheral analysis of the ankle joint, tDCS-treated rats had lower inflammation scores, that is, a reduced number of inflammatory cells, thus contributing with evidence on the mechanism of tDCS effects indexed by top-down activation[35,36]. It is important to note that this is the first study on

tDCS treatment in ovariectomized rats subjected to chronic inflammation induced by CFA injection.

CFA-induced inflammation has been shown to increase the levels of cytokines and free radicals, and this "inflammatory soup" sensitizes nociceptive neurons, enhancing neuronal excitability, and leads to secondary hyperalgesia[37]. It should be noted that CFA-induced inflammatory response is biphasic, with an early (acute) and a late (chronic) phase[38]. Our study focused on the late phase of inflammation, which induces immunological events[39], including an increase in the levels of inflammatory mediators, such as calcitonin gene-related peptide, NGF, IL-1β, and TNF-α[40].

Intraplantar administration of CFA induced not only peripheral inflammation but also microglial cell activation and increased the expression of pro-inflammatory cytokines (IL-1β, TNF-α, and IL-6) in the lumbar spinal cord, brainstem, and forebrain[41]. In addition, differences in cytokine response after CFA injection have been found in the hypothalamus: Charles River rats responded with increased TNF-α, IFN-γ, and IL-6 production with the development of adjuvant-induced arthritis, whereas Harlan rats had high hypothalamic levels of these 3 cytokines under control conditions[42]. Another recent study using a similar CFA model in mice demonstrated elevated IL-1β, IL-6, and TNF-α levels in the serum and expression of these pro-inflammatory cytokines in the basolateral amygdala of CFA-injected mice[43]. Also, chronic systemic inflammation promoted neuroinflammation mainly induced by IL-1β-positive microglia in female rats[44]. A previous study by our research group showed that CFA-induced orofacial pain increased brainstem IL-6 levels and decreased brainstem IL-10 levels, suggesting that the pain model induced an imbalance in the inflammatory system regarding the structure analyzed[45]. Altogether these studies highlight that peripheral CFA injection induces a response of inflammatory signaling in the peripheral and central nervous system.

This study has some limitations. All female rats underwent ovariectomy, and there was no ovariectomized group without tDCS treatment. Also, all rats were subjected to a model of chronic inflammatory pain. In addition, we did not use a positive control anti-inflammatory agent to verify the inflammatory profile. However, some points highlighted above have been discussed in our previous studies[18].

In summary, based on our findings, bimodal tDCS had an effect on the central inflammatory axis, with a small effect on the peripheral site as evaluated by histology in the current study. However, further studies are encouraged to better understand the relationship of inflammatory diseases in postmenopausal women and the effects of tDCS on these 2 associated conditions.

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