ORIGINAL ARTICLE

Improvement of the interleukin 2 and tumour necrosis factor α release by blood leukocytes as well as of plasma cortisol and antioxidant levels after acupuncture treatment in women suffering anxiety

Lorena Arranz, León Siboni, Mónica De la Fuente

Department of Animal Physiology II, Faculty of Biological Sciences, Complutense University of Madrid, Spain

Summary

In the context of psychoneuroimmunology, which studies the complex interactions between the nervous system and the immune system, anxiety-causing situations affect the immune function leading to its impairment, whereas acupuncture provides the possibility of improving it via its action on the nervous system. Therefore, the aim of the present work was to study, in anxious adult women, the effect of an acupuncture treatment specifically designed to relieve their symptoms, on several parameters known to be altered in disturbed emotional situations. These include the release by blood leukocytes of the cytokines interleukin-2 (IL-2) and tumour necrosis factor-alfa (TNF-α), as well as the plasma cortisol levels and total antioxidant capacity. The results show regulation by acupuncture of all the studied parameters, as early as 72 hours after one single acupuncture session. Thus, the levels of IL-2, which were decreased in women with anxiety, increase following acupuncture, and the levels of TNF-α and cortisol, which were increased in these patients, decrease after the session. Therefore, acupuncture could exert its therapeutic action on anxiety by modulation of a number of parameters including those examined in the present work.

Key words: anxiety – acupuncture – cytokines – cortisol – total antioxidant levels – women

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✉ Mónica De la Fuente, Department of Physiology (Animal Physiology II), Faculty of Biological Sciences, Complutense University of Madrid, José Antonio Novais 2, 28040 Madrid, Spain

☎ 00 34 91 394 49 89
🖷 00 34 91 394 49 35
🖳 mondelaf@bio.ucm.es

INTRODUCTION

Presently, it is accepted that the neuroendocrine and the immune system show a bidirectional communication mediated by shared chemical messengers and receptors, and thus both are involved in the preservation of homeostasis and therefore of health (Blalock 1994). Accordingly, if one of these two systems suffers alterations, this influences the other, and therapeutic interventions on one of these systems have an effect on the other. In fact, psychoneuroimmunology is the field of research that deals with the complex interactions

between the central nervous system and the immune system, and how behaviour and stress can modify these interactions (Glaser 2004). In this context, it is known that emotional disturbances lead to impairment of the immune system in individuals suffering from them (Herbert and Cohen 1993).

The neuroendocrine system of most interest in the study of stress and health is the hypothalamic pituitary adrenocortical (HPA) system, being in its secretion of cortisol the principal modulator of emotions, specially fear and anxiety (Müller et al. 2002). Immune cells exhibit receptors for many neuroendocrine products of the HPA axis, such as cortisol, which can cause changes in several of their functions, including the secretion of cytokines (Padgett and Glaser 2003). Glucocorticoids are able to modulate the pattern of activation of T-helper (Th) cells (principally $CD4^+$ cells), leading the immune system from cellular toward humoral responses (Zavala 1997), due to their ability to down-regulate the Th1 subset and to concomitantly up-regulate the Th2 subset (Rook et al. 1994). Since depression is often associated with alterations in HPA regulation, including increased plasma cortisol levels (Bauer et al. 2003), the suppressive effect of chronic stress, depression and anxiety on several immune functions (Leonard and Song 1996, Koh 1998) could be explained on the basis of the increased levels of that hormone. In this respect, several authors have shown that untreated patients with anxiety disorders exhibit significantly reduced lymphocyte proliferative responses to phytohemagglutinin (PHA) and concanavaline A (Con A) (Koh and Lee 1996, Leonard and Song 1996) and natural killer (NK) cell activity (Borella et al. 1999, Segerstrom et al. 1999). Moreover, a decreased PHA-induced production of IL-2, which is a cytokine related to lymphocyte proliferation and NK activity, has been described in anxious patients (Koh and Lee 1996), whereas pro-inflammatory cytokines, such as TNF-α, appear to be increased in those patients (Sluzewska et al. 1996).

Besides, reactive oxygen species (ROS) produced by leukocytes are important for host defence (Finkel and Holbrook 2000). However, an excessive production and / or release of ROS, accompanied by low levels or activity of antioxidant defences able to neutralize them, leads to a condition of oxidative stress, which is deleterious for cell homeostasis and function (Víctor et al. 2004, De la Fuente et al. 2005). Therefore, adequate amounts of neutralizing antioxidants are required to prevent damage.

Acupuncture is an ancient therapeutic practice widely used in Eastern countries, because of the favourable results obtained in the treatment of many diseases and pain relief (Richardson and Vincent 1986). It acts through stimulation of sensory nerves (Holaday 1983) and the autonomic nervous system (Nishijo et al. 1997), as well as via modulation of the release of hormonal factors, including endogenous opioids (Joos et al. 2000). More concretely, acupuncture may cause the release of β-endorphin into the circulation (Pomeranz 1976). However, it is unlikely that opioids in the periphery are analgesically active (He 1987), but they probably have effects on nonanalgesic systems, including the immune system (Mittleman and Gaynor 2000). In addition to β-endorphin, there are other hormones and neuropeptides that have been found to be released during acupuncture treatment (Bucinskaite et al. 1996). Thus, several authors have described changes in ACTH, leu- and met-enkephalin, dynorphin, serotonin and biogenic amines, among others (Mittleman and Gaynor 2000). Rather few, but interesting reports on acupuncture and its possible benefits in altered states of the nervous system, such as anxiety, are available (Sher 1998). In fact, it is well-known that anxiety disorders are related to endogenous opioid system deficits (Sher 1998).

In recent years, it has been proposed that acupuncture provides the possibility of influencing the immune system through its effects on the nervous system. In this respect, it has been reported that acupuncture may have favourable effects in the treatment of diseases that involve the immune system, i.e., allergic and inflammatory processes or different traumatisms (Kasahara et al. 1993, Joos et al. 2000). Acupuncture tends to normalize the circulating concentrations of leukocytes when they are disturbed (Mori et al. 2002). Furthermore, acupuncture regulates lymphocyte proliferation (Bianchi et al. 1991) and stimulates NK activity (Sato et al. 1996, Yu et al. 1998).

In view of the above, the aim of the present work was to study the effect of an acupuncture treatment on several parameters, which are known to be deteriorated in anxiety disorders, such as plasma cortisol levels and release of cytokines (IL-2 and TNF-α) by blood leukocytes, and on another parameter that might be altered in subjects with anxiety, such as the plasma total antioxidant capacity.

METHODS

Subjects

The study was performed in 27 female patients, aged 44.07 ± 2.18 years, who were suffering high levels of anxiety, as determined by the Beck Anxiety Inventory (BAI; Beck 1993). The control group was formed by 27 healthy women of approximately the same age (45.29 ± 1.88 years), to avoid gender and age related differences.

All subjects were Spanish and recruited from the population of Madrid. None of them received any kind of psychopharmacological treatment or other psychotherapy. Exclusion criteria consisted of malnourishment, pregnancy, severe allergics, immunodeficiency disease, neoplasic and autoimmune diseases, rheumatic fever, diabetes, scizures, endocrine disorders, anaemia, radiation therapy, chemotherapy, taking adrenal corticosteroids or estrogen replacement therapy, smoking more than 20 cigarettes per day, consuming alcohol or drugs and having performed endurance training shortly before admission. Women who had recently been diagnosed with infectious diseases, undergone surgical treatment, consumed immune-modulating drugs and/or showed pathologic laboratory findings were also excluded.

All participants received information about the purpose of the study and they gave their written consent for their blood samples to be used for academic research. All procedures were carried out according to the Declaration of Helsinki. Blood samples (9 ml drawn by vein puncture from peripheral blood) were collected from 8:00 a.m. to 9:00 a.m. (in tubes with citrate, BD Vacutainer Systems), in order to control the effect of circadian variations on the studied parameters. A clinical interview was followed by the performance of the psychological questionnaire (Spanish version of the BAI). Blood samples were collected afterwards.

The BAI is a 21-item scale concerning anxiety, especially in its physical aspects, during the past week including the present moment. It has a test-retest reliability of r=0.67–0.93 and its internal consistency is r=0.90–0.94.

Acupuncture treatment
The acupuncture protocol to treat anxiety consisted in needle stimulation of 19 acupoints, including ST36, LI4 and LI11, which have been related to immune functions and are often used in clinical practice for disorders of the immune system (Mori et al. 2002, Kou et al. 2005). Chinese needles (0.25 mm diameter), which were introduced to 10 mm, depending on the muscular mass, and Korean needles (0.16 mm diameter), introduced to a depth of 4 mm, were used. Each session lasted 30 min. Before the start of each treatment course and 72 hours after receiving one single acupuncture session, peripheral blood samples were drawn from all patients. Samples from controls were drawn only once.

Assay of TNF-α release
TNF-α release was measured on culture supernatants of total blood in the presence of LPS, following a method previously described (Hernanz et al. 1996). Briefly, 500 µl of blood were diluted 1:1 with RPMI-1640 medium without L-glutamine (Gibco, Burlington, Ontario, Canada) and incubated with 10 µl gentamicin 1% (1 mg/ml Gibco) and 10 µl LPS (250 ng/ml) for 4 h. Samples were centrifuged, and supernatants collected and frozen at –20 °C until assay. The TNF-α level was measured using an ELISA kit (DIACLONE Research, Besançon, France), with a minimum detectable dose of TNF-α less than 10 pg/ml. The results were expressed as pg/ml.

Assay of IL-2 release
Level of IL-2 was determined in the supernatants of peripheral blood lymphocyte cultures in the presence of PHA, following a method previously described (De la Fuente and Víctor 2000), after cell obtention. Aliquots of 200 µl lymphocyte suspension (10^6 cells/ml complete medium, containing 1640 RPMI, Gibco, Burlington, Ontario, Canada, supplemented with 1% gentamicin, 1 mg/ml Gibco, and 10% fetal calf serum, PAA, previously heat-inactivated, 30 min at 56ºC) were dispensed into 96 well plates (Orange Scientific, Belgium) and 20 µl/well PHA (Flow Laboratories) at 25 µg/ml were added. After 48 h of incubation, supernatants were collected and frozen at –20 °C until assay. IL-2 was measured using an ELISA kit (DIACLONE Research, Besançon, France), with a minimum detectable dose of IL-2 less than 10 pg/ml. The results were expressed as pg/ml.

Assay of plasma cortisol
The level of plasma cortisol was determined using a competitive immunoenzymatic colorimetric method for quantitative determination (BIOLINK, Barcelona, Spain). The inter- and intra-run precision had a coefficient of variation of 3.2% and 5.8% respectively. The values were expressed as ng/ml.

Assay of plasma total antioxidant capacity.
Antioxidant levels in plasma samples were evaluated with a kit provided by DELTACLON (Madrid, Spain), which is based on the reduction of Cu^{2+} to Cu^+ by the action of all present antioxidants. The amount of Cu^+ is determined through measuring the complex formed by Cu^+ and bathocuproine (BC). This complex is stable and has a typical absorption at 480–490 nm. The inter- and intra-run test precision had a coefficient of variation of 2.2% and 4.2% respectively. The results were expressed as units of total anti-oxidative capacity (unit/ml plasma). One unit of total anti-oxidative capacity is defined as the increase in the absorbance value of the reaction system in 0.01 per minute in each ml plasma at 37ºC.

Statistical analysis
Data are given as mean ± SEM of the values from the number of experiments shown in the figure

legends. Since the data were normally distributed (as shown by the Kolmogorov-Smirnov test), differences between patient and control groups were assessed by the Student's *t*-test for unrelated samples, and differences inside the patient group,

due to the treatment, were determined by the Student's *t*-test for related samples at the significance level 2α.
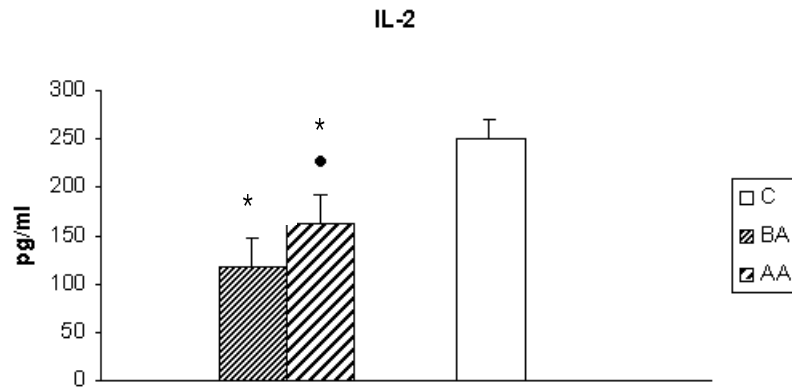
IL-2

Fig. 1. **IL-2 release (pg/ml) in lymphocyte culture supernatants of human peripheral blood lymphocytes from women suffering anxiety, before and 72 hours after receiving one single acupuncture session (patients) and from healthy women of the same age (controls).** Each column represents the mean ± SEM of 20 subjects, each value being the mean of duplicate assays.
* statistically significant with respect to the value in the control group
• statistically significant as compared to the value in those patients BA
AA: after the acupuncture treatment (72h after one single acupuncture session)
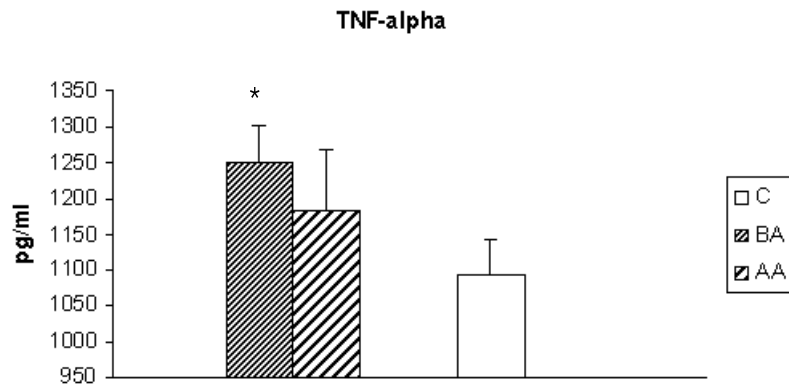BA: before the acupuncture treatment
C: controls

TNF-alpha

Fig. 2. **TNF-alpha release (pg/ml) in total blood culture supernatants in the presence of LPS from women suffering anxiety, before and 72 hours after receiving one single acupuncture session (patients) and from healthy women of the same age (controls).** Each column represents the mean ± SEM of 21 subjects, each value being the mean of duplicate assays. Symbols as in Fig. 1.

RESULTS

Cytokine release
IL-2 release in lymphocyte culture supernatants (Fig. 1; pg/ml), which was decreased (statistically significant) in anxious women (118±29) with

respect to controls (251±19), appears to be stimulated (statistically significant) 72 h after one single acupuncture session (162±31).

As regards TNF-α release, measured on culture supernatants of total blood in the presence of LPS (Fig. 2; pg/ml), which was increased

(statistically significant) in anxious patients (1,248±54) as compared to the control group (1,094±49), it appears to be similar to the control value 72 h after one single acupuncture session (1,118±83).

Plasma measurements
The level of plasma cortisol (Fig. 3; ng/ml), which was increased (statistically significant) in women suffering anxiety disorders (37±6) in comparison to controls (12±1), is lowered by acupuncture (28±4) 72 h after one single session (statistically significant).

With regard to plasma total antioxidant capacity (Fig. 4; unit/ml), women suffering anxiety disorders (223±11) showed diminished levels (statistically significant) when compared to healthy women (260±14), which are decreased (statistically significant) 72 h after one single acupuncture session (204±8).
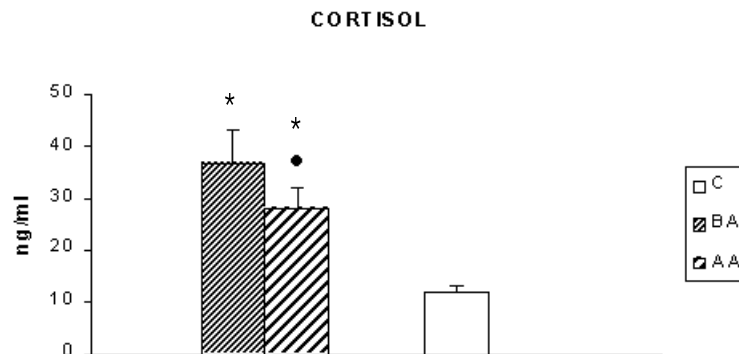

Fig. 3. **Plasma cortisol levels in women suffering anxiety, before and 72 hours after receiving one single acupuncture session (patients) and in healthy women of the same age (controls).** Each column represents the mean ± SEM of 25 subjects, each value being the mean of duplicate assays. Symbols as in Fig. 1.
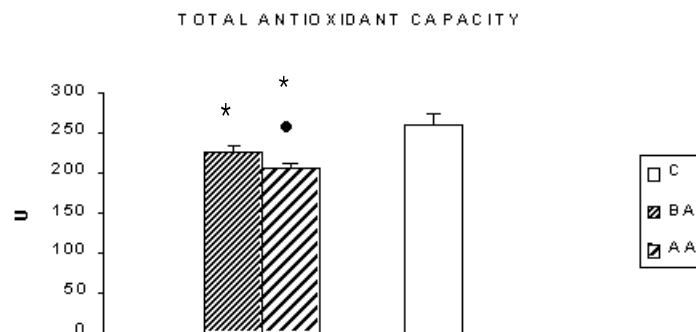

Fig. 4. **Plasma total antioxidant capacity in women suffering anxiety, before and 72 hours after receiving one single acupuncture session (patients) and in healthy women of the same age (controls).** Each column represents the mean±SEM of 27 subjects, each value being the mean of duplicate assays. Symbols as in Fig. 1.

DISCUSSION

The aim of the present work was to study some key parameters known to be deteriorated in anxious women as compared to those of healthy subjects, such as blood leukocyte cytokine release (IL-2 and TNF-α) and plasma cortisol levels, as well as a parameter which might also be altered in those patients, namely plasma total antioxidant capacity, and to determine the effect on these parameters of an acupuncture treatment designed specifically to relieve the symptoms derived from anxious disorders. Since Bossy (1990) indicated that the response to one single acupuncture session frequently shows a 12–24 hours delay and remains for 5–7 days, we analyzed these parameters 72 hours after one single acupuncture session. We used similar data with regard to age and gender in control and patient groups to avoid any variability in the studied parameters caused by those factors. It must also be considered that, although the session itself may influence both the subjective status of the subjects and a number of their measured data, several placebo-controlled studies on the effects of acupuncture on immune functions have shown significant improvements in patients treated with real acupuncture with respect to those who received sham acupuncture (Karst et al. 2003, Kou et al. 2005). Thus, the control group used in the present work was formed by healthy women who were not treated at all.

Regarding cytokine results, the decreased PHA-induced IL-2 levels in women suffering anxiety disorders, are stimulated by acupuncture, even 72 hours after receiving the session. Since cytokines are crucial modulators of the complex network of cellular interactions that regulate the immune response, changes in their content could play an important role in the immune function impairment observed in anxiety disorders. In this respect, IL-2 is an important stimulus for lymphoproliferative responses and NK activity (Solana et al. 1999), and thus its decrease could be related to the decline of those functions, which has been found in anxiety disorders (Koh and Lee 1996, Borella et al. 1999, Segerstrom et al. 1999). Further, acupuncture could exert some of its immunomodulatory action through the regulation of cytokine release, including IL-2. In fact, other authors have reported the stimulation of IL-2 release by acupuncture in a variety of experimental protocols (Yang et al. 2003, Qui et al. 2004).

As regards LPS-induced TNF-α, pro-inflammatory cytokine which is increased in anxious women, it appears to be decreased to control levels 72 hours after one acupuncture session. Pro-inflammatory cytokines and oxidant compounds produced during the inflammatory response, which follows infection, may be beneficial or deleterious, depending on the amounts

and circumstances in which they are produced. Although pro-inflammatory molecules are necessary for host defence, especially against pathogen microorganisms, excessive levels could be detrimental, since they are related to chronic inflammatory diseases, infectious processes, and autoimmune diseases (Grimble 1998), among others. Therefore, acupuncture may be beneficial in anxious emotional situations, decreasing and restoring the TNF-α levels to healthy values. Similar results have been described dealing with other pathological conditions (Jeong et al. 2002).

Cortisol is the main glucocorticoid which modulates emotions, such as anxiety (Müller et al. 2002), and is one important factor that may contribute to the immune function decline observed in anxious disorders. In fact, it is well known that cortisol can change immune functions such as cell trafficking, proliferation, cytokine secretion, antibody production and cytolytic activity (Madden 2001, Padgett and Glaser 2003). The increased levels of plasma cortisol found in women with anxiety are decreased by acupuncture even 72 hours after the session. Other authors have reported decreasing plasma cortisol levels after acupuncture treatment (Han et al. 2004). Therefore, acupuncture could regulate the immune system through the modulation of altered cortisol levels.

The present results have shown that the plasma total antioxidant capacity was decreased in anxious emotional situations, reflecting a reduced capacity to rapidly handle an increase of ROS. Since immune cell functions are especially linked to generation of these species, those functions would be compromised by the imbalance between oxidants and antioxidants (Berlett and Stadtman 1997). Moreover, immune cells are particularly sensitive to oxidative stress because of the presence of polyunsaturated fatty acids in their plasma membranes (Izgüt-Uysal et al. 2004). Additionally, the immune system is highly reliant on accurate cell to cell communication, thus damage to it results in an impaired general immune responsiveness (Víctor et al. 2004). In this respect, the presence of oxidative stress in other mental disorders, such as schizophrenia (Reddy et al. 2003), has been suggested by the finding of decreased plasma total antioxidant capacity. In fact, the determination of plasma total antioxidant capacity has been proposed in order to assess the level of oxidative stress in a variety of situations (Aycicek et al. 2005). Interestingly, this capacity appears to be decreased by the acupuncture treatment of anxiety 72 hours after one session, which suggests antioxidant internalization by blood cells, including immune cells. In this respect, it is well known that a significant contribution to the body's total antioxidant capacity comes from antioxidant molecules present in plasma (Reddy et al. 2003), because plasma is an important vehicle

that protects blood components from oxidative damage and distributes dietary antioxidants to the rest of the body. Several authors have reported changes in the antioxidant status caused by acupuncture (Pogosyan et al. 2004, Spence et al. 2004), and Pogosyan et al. (2004) reported an improvement of the antioxidant status of erythrocytes after acupuncture treatment. Thus, acupuncture could be decreasing the oxidative stress affecting immune cells, through the increase of intracellular antioxidant levels, and as a result, improving the function of these cells.

In view of the above, acupuncture could exert its beneficial effect on the impaired immune function accompanying anxiety disorders, at least through modulation of cytokines such as IL-2 and TNF-α, cortisol levels and antioxidant status of immune cells.

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