

Effect of acupuncture on the neutrophil respiratory burst: a placebo-controlled single-blinded study

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SUMMARY. Objectives: Little is known about the influence of acupuncture treatment on the phagocytic immune system. This trial was performed to examine whether multiple acupuncture treatment affects the respiratory burst (RB) of neutrophils, a recognised measure of their cytotoxicity. Design: Placebo-controlled single-blinded study. Interventions: Eleven volunteers were treated bilaterally with standard needles (real) at acupoint LI11, 11 volunteers with placebo needles (placebo) at the same point. Treatments were performed for 30 min each twice a week for 4 weeks, eight times in all. The standard needles were manipulated until needle sensation (DEQI) developed. Before the treatment course (baseline), 48 h after the fourth (follow-up 1) and 48 h after the last treatment (follow-up 2) blood samples were drawn. Main outcome measures: RB and plasma β -endorphin at each time point. Results: In the real group there was a highly significant increase in the RB at follow-ups 1 ($P = 0.004$) and 2 ($P = 0.007$). β -Endorphin levels decreased, but not significantly. In the placebo group there was a significant increase in the RB at follow-up 2 ($P = 0.048$). In addition, at follow-up 2 a significant drop in β -endorphin levels was observed ($P = 0.015$). Conclusions: The RB of neutrophils is significantly activated by a course of several acupuncture treatments. In addition, psychological effects and a placebo that was not totally inert may contribute to the findings in the placebo group which may be mediated by the opiate endorphin system. © 2003 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

An increasing proportion of the population in many countries of the Western world perceive complementary medicine, such as acupuncture, as a safer alternative for non-life threatening conditions, such as common colds, cough and some inflammatory conditions, even though knowledge of the mechanisms of acupuncture activity remains inconclusive or incomplete.

Acupuncture treatment appears to have the potential to modulate hormonal factors, including endogenous opioids¹⁻⁵ and the autonomic nervous system.⁶

Lymphocytes and polymorphonuclear leukocytes (PMNs), such as neutrophils, possess both opioid receptors and β -adrenergic receptors.^{7,8} Evidence has been found that acupuncture increases CD3⁺ cells, CD4⁺ cells^{1,9} and CD8⁺ cells.⁹ A few studies¹⁰⁻¹² have also come to the conclusion that the phagocytic activity of macrophages and the phagocytic activity and migration of neutrophils are improved

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after acupuncture treatment. Therefore, acupuncture may have an influence on the immune system or on its components. In almost all studies investigating the immunological effects of acupuncture, acupoint LI4 has been used, usually in combination with other acupoints, depending on the underlying diseases which were to be treated. LI4 and LI11 are both located on the LI-meridian and are supposed to be strong "immunomodulatory" acupoints which eliminate "heat" in inflammatory conditions.¹³ Puncturing at the latter point is easily performed and may be suitable for weekly self-application.

A serious problem in such studies is that the immune system may be affected by psychological factors which may influence the CNS and the endocrine system. It is, therefore, essential to use a placebo method of needling with the same psychological impact as actual needling. Streitberger et al., Park et al. and our own group have developed a placebo needle in which a blunt needle in a mounting support strikes the skin without penetrating it. This placebo needle apparently allows a valid assessment of the placebo effect of needle tip penetration of acupuncture treatment.¹⁴⁻¹⁶ In a study¹⁷ testing the credibility of the placebo needle device, no difference between real and placebo acupuncture was detected.

PMNs are the first line defence against bacterial and fungal infections. Adherence, chemotaxis, phagocytosis, and the activation of intracellular enzyme systems, such as the respiratory burst (RB), are the major components in the PMNs' arsenal against microbial infection. The production of reactive oxygen intermediates (ROI) during the RB is one of the main mechanisms used by PMNs for bacterial and fungal elimination.^{18,19} Rothe et al.²⁰ have shown that the production of ROI during RB can be quantified in real-time with multiparameter flow cytometry (MFC) using the dye dihydrorhodamine 123 (DHR) which reacts with free radical-derived oxidants to become the brightly green fluorescent rhodamine 123 (rho-123). MFC simultaneously assesses five variables per measured event: relative cell (particle) size, density (granularity) and three fluorescences, representing surface, intracellular or cell cycle characteristics. Compared with chemiluminescence, which estimates only non-specific oxygen radical generation, the methodology of this study has the advantage of assessing specific intracellular oxygen radical production by plasma membrane RB oxidase of a certain cell population, such as neutrophils. Using this method the ROI production of erythrocyte-depleted whole blood samples can be measured without further time-consuming purification steps. Possible harmful manipulation of the isolated cells can also be avoided and highly reproducible and reliable results are obtained in the minimum of time.²¹

The aim of the present placebo-controlled study was to examine whether repeated chronic bilateral acupuncture treatments on acupoint LI11 have a specific effect on neutrophil phagocytic activity, measured as the RB, and whether there is a simulta-

neous change in the plasma levels of β -endorphin, an endogenous opioid which is believed to affect immunological function.²²

MATERIAL AND METHODS

The study protocol was approved by the institutional ethics committee, and written informed consent was obtained from all volunteers. The 22 volunteers (10 male, 12 female, mean age 31.86 years (range 20-48)), all health care professionals without any organic or psychiatric disease, were randomised into real and placebo groups. They were told that acupuncture treatment may have the power to modulate the immune system and that we planned to examine this with a course of eight acupuncture treatments. The treatments were carried out in such a way that the volunteers of the different groups had no contact with each other. Volunteers in the placebo group were told that the needles would only be stuck in gently and superficially and that an elastic cube would, therefore, be necessary to fix the needle. Questions from the volunteers were prevented by instructing them to relax throughout the treatment and that this was particularly important for the effectiveness of acupuncture. At the first treatment the volunteers were told that they may feel a prick from the needle. They were also told that a DEQI sensation may occur when the needle is inserted, and that this is a feeling of numbness which may be accompanied by warmth and may spread from the site of puncture.

The real group was treated with standard needles (Seirin B-type needle No. 8 (0.3 mm \times 0.3 mm)) at acupoint LI11 (lateral end of the elbow fold) bilaterally, the placebo group with placebo needles (see the following description) at the same point. Treatments were performed for 30 min each, twice a week, eight times in all. The standard needles were manipulated until needle sensation (DEQI) was felt. Before the treatment course (baseline), 48 h after the fourth (follow-up 1) and 48 h after the last treatment (follow-up 2), blood samples were drawn and the RB was determined. In addition, plasma β -endorphin was determined at the same time points.

For measurement of RB and β -endorphin, venous blood samples (7.5 ml) were collected in lithium heparin-coated disposable blood sampling tubes (Sarstedt, Nuembrecht, Germany) and EDTA-treated venipuncture tubes (Sarstedt, Nuembrecht, Germany), respectively. While the RB was measured immediately the samples for the β -endorphin analysis were stored at -20°C and measured later.

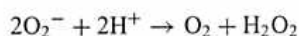
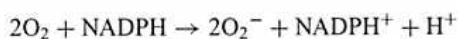
Placebo needle

For preparing the needle, the tip of a real acupuncture needle (No. 16, 30 mm \times 0.3 mm, Asiamed Inc., München, Germany) was removed and the new end was rounded with a diamond polisher. When it touches the skin a pricking sensation is felt by the volunteers, simulating puncturing of the skin. To

support the needle we used a cube of elastic foam which we fixed upon the area of the acupoint. In this way, the volunteer could not see that the blunt placebo needle is not inserted into deeper tissue layers. Because of the elasticity of the foam the needle appears to be shortened.

Sample preparations for the determination of the RB

Leukocytes were counted in a Neubauer cell counting chamber. The activity of the NADPH oxidase was measured by the intracellular oxidation of DHR (MoBiTec, Goettingen, Germany) to the fluorescent dye rho-123 in a flow cytometer. The assay depends upon the incorporation of DHR into the cell. After cell activation, the NADPH oxidase catalyses the reduction of oxygen to superoxide anion which is further transformed by dismutation to hydrogen peroxide. The non-fluorescent DHR is oxidised intracellularly in a peroxidase-dependent reaction to green fluorescent rho-123. The amount of rho-123 is proportional to generated H_2O_2 (see formula below).



After whole blood Ficoll sedimentation (Ficoll-Hypaque, density 1.077 g dl^{-1} , Biochrom, Berlin, Germany) the leukocyte supernatant (containing an average of $5 \times 10^5 \text{ cells ml}^{-1}$) was added and incubated with $1.65 \times 10^{-5} \text{ mol DHR}$ (1.1 mM , $15 \mu\text{l}$, MoBiTec, Goettingen, Germany). The RB was induced by either priming with 10 ng recombinant tumour necrosis factor alpha (TNF- α , $1 \mu\text{g ml}^{-1}$, $10 \mu\text{l}$, Sigma) for 5 min followed by receptor stimulation with $1 \times 10^{-7} \text{ mol N-formyl-methionyl-leucyl-phenylalanine}$ (FMLP, 0.01 mM , $10 \mu\text{l}$, Sigma) or the RB was induced by FMLP or by phagocytosis of $20 \mu\text{l}$ *Escherichia coli* ($1 \times 10^9 \text{ bacteria ml}^{-1}$, HB 101, Sigma). TNF- α /FMLP and *E. coli* were used in moderate doses in which either RB stimulating or RB reducing effects could be observed. After 20 min for viability discrimination dead cells were counterstained with $1 \times 10^{-5} \text{ mol propidium iodide}$ (PI, 1 mM , Serva, Heidelberg, Germany). Samples were stored on ice and measured in a flow cytometer within 30 min (see the following description). To detect possible pre-activation of neutrophils negative controls without stimulation were carried out.

Flow cytometry adjustment and acquisition

Both applied flow cytometric assays allow quantification of PMNs response at single cell level. The flow cytometer was equipped with an argon ion laser adjusted to a wavelength of 488 nm (Epics

XL[®], Beckman-Coulter, Krefeld, Germany). For each sample 20,000 events were measured. The rhodamine emission of the RB were measured with the photomultiplier of the green fluorescence (FL1, 515–545 nm). The photomultiplier for the red fluorescence (FL3, 650 nm) was used to measure PI emission for discrimination between vital and necrotic cells. Side scatter (SSC) and forward scatter (FSC) were assessed in linear mode; FL1 and FL3 in logarithmic mode without compensation. The photomultiplier volts and gains of FSC, SSC, FL1 and FL3 were adjusted for each negative control and remained constant for the matched samples. Data files were stored in list mode and analysed in dot plots using a PC-software package (EXPO[®] 2.0, Beckman-Coulter).

Analysis of RB

Erythrocytes and cell debris were excluded according to a discriminator adjusted in the FSC signal. Neutrophils were included by setting a polygonal gate in an FSC/SSC dot plot. These gated cells were transferred to an SSC/FL3 dot plot for exclusion of dead neutrophils, which due to their high fluorescence in FL3 result from the intracellular PI content. Finally, only vital neutrophils were included in an SSC/FL1 dot plot. The percentage of rhodamine positive neutrophils was calculated after setting a quadrant region in the SSC/FL1 dot plot of the negative control (Fig. 1).

β -Endorphin detection

The quantitative assessment of β -endorphin was carried out with the two-site immunoradiometric assay (IRMA) produced by Nichols Institute Diagnostics (San Juan Capistrano, USA). This test uses a specific anti- β -endorphin antibody, coated on a plate to which the serum sample to be determined is added. After ^{125}I -labelled second antibody has been added and the plate rinsed, the radioactivity is measured by a gamma-counter. The concentration of β -endorphin is proportional to the radioactivity measured and it is related to a standard curve obtained with the same method. Specimen collection, preparation and assay procedure were performed as described in the immunoassay kit.²³

Statistical analysis

The sample size required to achieve 80% power for the expected differences between mean values was calculated as 11 for each group.

The percentage of activated neutrophils (RB assay: rhodamine positive neutrophils after induction with *E. coli*, TNF- α /FMLP or FMLP) 48 h after four (follow-up 1) and eight (follow-up 2) needle acupuncture treatments with standard or placebo needles was compared to the percentage of activated neutrophils before acupuncture treatment (baseline).

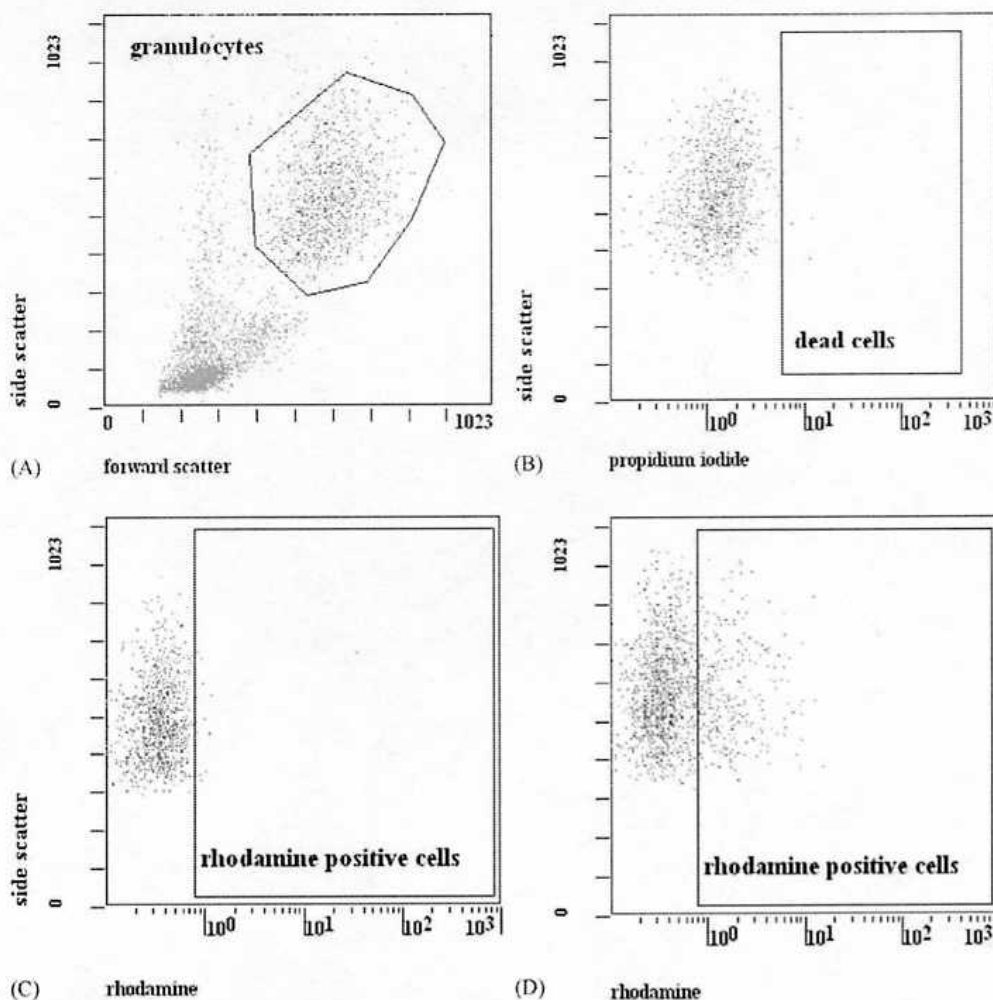


Fig. 1 Adjustment of the acquisition dot plots for analysis of the neutrophil respiratory burst due to the green rhodamine fluorescence. (A) Forward scatter (FSC) vs. side scatter signals (SSC) were depicted as acquired on the flow cytometer. Neutrophils were localised and gated due to their high SSC signals. Only neutrophils from this region were inserted to the next dot plot. (B) SSC vs. red propidium iodide fluorescence (photomultiplier FL3) for gating of necrotic neutrophils (dead cells) due to their high red fluorescence. Cells from this gate were excluded for the following dot plots. (C) and (D) SSC vs. green rhodamine fluorescence (photomultiplier FL1) for gating of rhodamine positive cells using the gate combination "granulocytes" of dot plot A but not "dead cells" of dot plot B. Dot plot C is an example for a negative control without stimulation, whereas dot plot D depicts RB positive PMNs after TNF- α /FMLP stimulation

Results are presented as mean \pm standard deviation (S.D.). Time-dependent intra-group data were evaluated using the two-tailed, paired *t*-test; inter-group data were evaluated by computing the difference and using the two-tailed, unpaired *t*-test. A probability of less than 0.05 was considered significant.

RESULTS

There was no difference between the two groups in age and sex (Table 1). One hundred percent of the volunteers felt DEQI when acupuncture with standard needles, but only 54.5% with the placebo needles (Table 1). Because of an error in sample

Table 1 Baseline values (age, sex, DEQI, and mean percentage of the activated neutrophils (PMNs) and plasma β -endorphin levels)

	Real acupuncture (n = 11)	Placebo acupuncture (n = 11)
Age (years)	31.09 \pm 8.34	32.64 \pm 8.77
Sex (male/female)	5/6	5/6
DEQI (N)	11	6
TNF- α /FMLP (%)	24.02 \pm 7.74	24.51 \pm 9.57
<i>Escherichia coli</i> (%)	41.22 \pm 8.80	42.43 \pm 11.71
β -Endorphin (pg ml $^{-1}$)	24.67 \pm 15.98	32.91 \pm 12.23

Table 2 Mean percentage of the activated neutrophils (PMNs) and plasma β -endorphin levels before acupuncture treatment and at follow-ups 1 and 2 for the real group (R) and the placebo group (P)

		Baseline (before treatment)	Follow-up 1 (48 h after four treatments)	Follow-up 2 (48 h after eight treatments)
TNF- α /FMLP (%)	R	24.02 \pm 7.74	36.05 \pm 8.70 ^{**†}	42.89 \pm 17.88 ^{**}
	P	24.51 \pm 9.57	23.32 \pm 9.84	31.51 \pm 7.58 [*]
FMLP (%)	R	10.97 \pm 5.36	11.89 \pm 4.80	13.22 \pm 6.58
	P	9.44 \pm 4.36	8.66 \pm 2.85	12.64 \pm 5.10
<i>Escherichia coli</i> (%)	R	41.22 \pm 8.80	52.44 \pm 14.70	45.43 \pm 6.27
	P	42.43 \pm 11.71	41.39 \pm 11.61	49.34 \pm 10.72 [*]
Plasma β -endorphin (pg ml ⁻¹)	R	24.67 \pm 15.98	29.29 \pm 17.61	17.97 \pm 8.59
	P	32.91 \pm 23.23	34.14 \pm 14.66	28.99 \pm 9.53 [*]

* $P < 0.05$.
** $P < 0.01$.
† Inter-group difference $P < 0.05$.

preparation there was a drop-out of one sample in the real group.

In the real group eight volunteers had rises in the RB at each follow-up referring to the individual basal levels after stimulation of the neutrophils with TNF- α /FMLP, two showed no changes at all. This was a highly significant increase in the mean values of the RB: from 24.02 \pm 7.74 to 36.05 \pm 8.70% ($P = 0.004$) at follow-up 1 and 42.89 \pm 17.88% ($P = 0.007$) at follow-up 2. After stimulation with *E. coli*, the mean values of the RB at the two follow-ups were greater than the mean initial value, although this was not statistically significant. The β -endorphin levels dropped from 24.67 \pm 15.98 to 21.29 \pm 17.61 pg ml⁻¹ ($P = 0.110$) at follow-up 1 and 17.97 \pm 8.59 pg ml⁻¹ ($P = 0.103$) at follow-up 2. These changes were not statistically significant (Table 2).

In the placebo group seven volunteers (four with DEQI, three without DEQI) had some rises in the RB at each follow-up referring to the individual basal levels after stimulation of the neutrophils with TNF- α /FMLP, four showed no changes at all. This was a significant increase in the mean values of the RB at follow-up 2, from 24.51 \pm 9.57 to 31.51 \pm 7.58% ($P = 0.048$). After stimulation with *E. coli* there was a significant increase in the mean values of the RB at follow-up 2, from 42.43 \pm 11.71 to 49.34 \pm 10.72% (0.039). In addition, at follow-up 2 a significant drop in β -endorphin levels was observed, from 32.91 \pm 23.23 to 28.99 \pm 9.53 pg ml⁻¹ (Table 2).

The increase in the RB after stimulation with TNF- α /FMLP was significantly greater in the real group in comparison with the placebo group only at follow-up 1 ($P = 0.024$). Other significant differences between the real and placebo groups were not found (Table 2).

DISCUSSION

The phagocytic activity of macrophages and the phagocytic activity and migration of neutrophils af-

ter acupuncture treatment have been measured in a few studies.¹⁰⁻¹² although there is no conclusive evidence for a specific acupuncture effect, as a result of methodological weaknesses in the design of the studies and lack of statistical significance in the results. In an uncontrolled trial, Sliwinski and Kulej¹¹ showed that neutrophil migration returned towards normal in most patients with severe chronic bronchitis during the course of 42 treatments. No details of the clinical condition of the patients were given. Additionally, all patients had been taking corticosteroids for up to 24 years and these were stopped when the trial began. This may be one more reason for the improvement in neutrophil migration. Zhou et al.¹² showed with a controlled study of postoperative patients who received strong stimulation to two acupoints daily for 3 days that the phagocytic activity of the neutrophils in the treatment group significantly increased, though not the lymphocyte counts. The controls were not treated at all.

Since the most accurate tests for the state of the immune system are measurements of function we used the determination of the neutrophil RB, a measure of phagocytic activity, as main outcome parameter. Altogether our results show that given over several weeks, both real and placebo acupuncture produce significant changes in the neutrophil cytotoxicity, but that these changes were more marked and more rapid with real acupuncture. The changes in the mean values of the RB are more marked after the stimulation with the combination TNF- α /FMLP than with FMLP solely because of the priming effect of TNF- α on the activity of different receptors of the PMNs. In addition, we observed that β -endorphin plasma levels significantly decreased in the placebo group. However, the baseline β -endorphin levels of the placebo group were higher than those of the real group which makes interpretation difficult. One explanation for this finding may be that there exist huge individual differences in the β -endorphin plasma levels which did not balance each other out because of the small sample size.

We suggest that the physiological effects of acupuncture contribute significantly to these positive findings which are more intense in the real acupuncture group, as only here does penetration occur (100% DEQI). On the one hand, psychological effects depending on conditioning and on the other hand a placebo that was not totally inert may contribute to the findings in the placebo group in which 6 out of 11 felt DEQI (54.5%) but 3 without DEQI had marked rises in the RB. The volunteers *knew* treatment was being performed to improve the immune system. This knowledge could have had the effect of a hypnotic suggestion or a cognitive-behavioural intervention both which have been shown to modulate immune function.^{24,25} Conditioning is a well-known strategy to modulate immune function.^{24,25} For example, if a distinctly flavoured drink is given to an animal several times together with an immunosuppressant drug, subsequently the drink is able to suppress the immune system in the absence of the drug.²⁶

In pre-tests we did not find any immediate effect on the RB of the neutrophils 30 min after removal of the needles.²⁷ This is in good agreement with Bossy²⁸ who pointed out that the onset of a response to acupuncture is often delayed by 12–24 h and lasts for 5–7 days.

These effects may both be partially mediated by changes in the endorphin system, which were clearer in the placebo group. There is some previous evidence for an interaction between the endorphin system and neutrophil activity. In *in vitro* studies with white blood cells from both cancer patients and normal controls, both leu- and met-enkephalin enhanced neutrophil chemiluminescence (an index of phagocytic activity).²² Bianchi et al.²⁹ analysed the levels of opioid peptides within white cells and correlated them with immune activity. The levels of β -endorphin in the immune cells of patients with chronic low back pain were reduced before treatment; normal levels were restored after a course of seven treatments with acupuncture, although a single treatment was not sufficient.²⁹ These results are in satisfactory agreement with our finding of a decrease in the β -endorphin plasma level, if one postulates increased cellular uptake.

Although it is not yet clear to what extent these positive immunological changes translate into any concrete improvements in relevant aspects of health, it shows the synergistic power of the combination of psychological and physiological interventions.

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