Research Article

Repeated cupping manipulation temporary decreases natural killer lymphocyte frequency, activity and cytotoxicity

Boris V. Dons’koi1, Viktor P. Chernyshov1, Dariia V. Osypchuk1, Sergiy M. Baksheev2

1. Laboratory of Immunology, Institute of Pediatrics, Obstetrics and Gynecology, National Academy of Medical Sciences of Ukraine, 04050 Kiev, Ukraine
2. Gynecology Department, Kiev Maternity Hospital, 03148 Kiev, Ukraine

ABSTRACT

OBJECTIVE: Elevated natural killer lymphocyte cytotoxicity (NKc) has been linked with reproductive problems in women. Here we evaluate the potential benefit of cupping therapy (CT) in reproduction-related immune responses.

METHODS: This was a pilot clinical study. Participants were healthy female volunteers (n = 23) with elevated NKc, and received repeated CT 3 times over 5 d (inner pressure 40–50 kPa, 40 min; 12–15 cups). Lymphocyte subsets, NKc and NK lymphocyte activity (NKa) were measured in blood on day 0 (initial levels, before the first treatment) and days 3, 10 and 17 after the last CT treatment, using the K562-stimulated CD69 expression assay.

RESULTS: As a result of CT manipulations NKa was reduced on days 3 and 10, and NK percentage was reduced on day 10. NKc was most sensitive to CT treatment, resulting in their decreased counts at 3, 10 and 17 d post CT. CT treatment decreased NKc in the majority of individuals (87%), but the magnitude of the effect was variable. Out of 23 subjects 9 (39.1%) had a 2–3 fold decrease of NKc on days 3, 10 and 17; 11 (47.8%) started to show a decrease in NKc later, or more quickly returned to base levels; and only 3 (13%) subjects displayed no effect of CT on NKc. Expectedly, no changes in T-cell subsets (CD3CD4, CD3CD8, HLADR, CD158a) were observed after CT.

CONCLUSION: CT decreased NK cell numbers, their activity and cytotoxicity. Low cost, safety, non-invasive nature and ease of administration make CT a promising approach for NKc down-regulation.

Keywords: killer cells, natural; cytotoxicity; cupping therapy


1 Introduction

In the Western world cupping has been widely used in medical practice for the treatment for pain syndromes[1,2]. However, the precise mechanism of its action is currently unclear. Cupping therapy (CT) results in a strong increase of lactate and an associated shift in the lactate/pyruvate ratio, indicating anaerobic metabolism in the surrounding tissues[3]. Additionally, “cupping blood” had higher activity of myeloperoxidase, lower activity of superoxide...
dismutase, and higher levels of malondialdehyde and nitric oxide, compared to venous blood\(^4\). Cupping also causes prolonged exposure of the skin to very reduced pressure, leading to the hyper-dilatation of blood vessels and local blood congestion causing intravascular stasis\(^5\). It has also been shown that some variants of CT have modulatory effects on the innate and adaptive cellular immune responses\(^6\).

Nature killer (NK) lymphocytes play a key role in processes critical for human reproduction\(^7,8\). NK-excited cytotoxicity is one manifestation of an imbalance in the general function of the NK system and is associated with reproductive problems in women, such as implantation and pregnancy failures\(^9-12\). Thus, control of NK lymphocyte cytotoxicity (NKc) for therapeutic gain in the treatment of reproductive problems warrants a thorough investigation. This holds true since current methods, such as intravenous immunoglobulin or lipid infusion, lymphocyte immunization or intrauterine lymphocyte administration are still waiting to receive reliable evidential basis\(^13-16\).

The goal of this study is to identify how the peripheral blood cells are affected by CT treatment, and subsequently return to the general blood flow on the number, activity and cytotoxicity of NK lymphocytes. We hypothesize that: (1) Low pressure under the cups, for the duration of the treatment, stretches the skin, leading to the reshaping of skin vessels and subsequently filling them with blood. (2) This blood persists at the treatment site for 3–5 d, affecting other blood cells through contact, close interactions and acidification, resulting in activation and apoptosis. (3) Affected blood lymphocytes return to the general blood flow without inflammation, tissue damage and/or thrombosis. (4) The immune system (especially NK cells) spends its reserve of resistance to eliminate the affected cells. (5) Repetition of this procedure several times wears out NK system by exhausting its excess reserves.

The objective in the experimental model has been the controlled temporary suppression of NKc using CT.

## 2 Methods and patients

### 2.1 Study design

We conducted a pilot study using 23 healthy, 18–25 years old, female volunteers, without chronic or actual infection and with previously confirmed elevated NKc. Cups were applied (\(n=12\) cups) between days 5 and 20 of their cycle, three times (every other day) for 45 min (Figure 1). The pressure inside the cups was evaluated by visual inspection of the stretching of the skin, which occupied 25%–40% of the volume of cups (equivalent 40–50 kPa). The skin was treated with 30% glycerin solution prior to cupping in order to prevent damage

![Figure 1](image.png)

**Figure 1** Flow diagram of the present study
A: Flow diagram of blood examination and cupping therapy manipulations; B: Form and color of intravascular stases unchanged 30 min, 2 h, 48 h and 5 d after cupping therapy. For control, in presented variant, we treated one (upper) side of cupping trace by heparin ointment immediately after manipulation. We did not find any difference in shape and resorption time between the two sides. It confirmed absence of capillary hemorrhage in proposed modification of manipulation. CT: cupping therapy.
when stretched. Individuals were tested for lymphocyte subpopulations, NK lymphocyte activity (NKa) and NKc immediately before CT (initial levels on test 0) and on days 3 and 10 and (only some individuals) on day 17 after last CT manipulation. In a separate study we have repeated this study protocol in the same individual one year later to compare the type response (Figure 2). All of the study subjects signed an informed consent form before being enrolled in the study (approved by the Biomedical Ethics Committee of Institute of Pediatrics, the Obstetrics and Gynecology, the National Academy of Medical Sciences of Ukraine №6 17/12/2012 and the Clinical Immunology and Allergology Committee of Ministry of Health №11 14/02/2013). Three individuals were excluded from the analysis for the following reasons: one had a viral respiratory infection, another had a case of herpes simplex and the third subject had premature menstruation after first CT manipulation. Twenty-three individuals received three CT manipulations, and peripheral blood was analyzed on the 3rd, 10th and 17th day after CT.

2.2 NKa measurement
NKa was investigated as described previously\(^\text{[17]}\). Specifically, 0.1 mL of freshly collected blood was coincubated with or without 0.1 mL containing \(2.5 \times 10^6\) freshly washed K562 cells (an human leukocyte antigen-negative erythroleukaemia cell line). Cells were stained with 20 \(\mu\)L/tube of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and PE-Cy5-conjugated monoclonal antibodies against CD69, CD56 and CD3 (BD Bioscience, San Jose, CA, USA), lysed, washed, and analysed using a FAC Scan flow cytometer and CellQuest software (BD Bioscience, San Jose, CA, USA). Using these data, the frequency of NK (NK%) as a percentage of CD56/CD3 lymphocytes and the frequency of CD69 NK cells were calculated.

2.3 Flow cytometry of lymphocyte subsets
Lymphocyte subsets were identified by three-color flow cytometry, using the erythrocyte-lysing whole blood method of lymphocyte staining by FITC-, PE- or PE-Cy5-conjugated monoclonal antibodies (BD Bioscience, San Jose, CA, USA). Stained samples were lysed, washed and analyzed by an FAC Scan flow cytometer using CellQuest software (BD Bioscience, San Jose, CA, USA). In both studies we analyzed the expression of HLADR, CD158a, CD69 and CD8 in CD3\(^+\), CD3\(^+\)CD4\(^+\), CD3\(^+\)CD8\(^+\), CD3\(^+\)CD56\(^+\) and CD3\(^+\)CD56\(^-\) lymphocytes.

2.4 NKc assay
NKc assay was conducted using a flow cytometry

![Figure 2](image-url)

**Figure 2** Reproducibility of cupping therapy effect on natural killer numbers (NK%) and natural killer cytotoxicity (NKc)
Dynamics of parameters without cupping treatment (A), after first cupping therapy (B), and after second cupping therapy (C) are shown in the figure. Data were obtained from two cupping therapy manipulation on the same individual.
described previously\textsuperscript{(17)}. We used carboxyl methyl fluorescein double acetate (Molecular Probes, Eugene, WA, USA) for labeling K562 as target cells. Dead cells after a 4-hour incubation were labeled by propidium iodide. Less than 5% spontaneous lysis of target cells was observed in these experiments.

2.5 Statistical analysis

The statistical analysis of the results was performed using approximation of Woolf for odds ratio (OR) and 95% confidence intervals (CI), and Fisher’s exact test (two-side P-value) (In Stat version 3.0 for Windows Graph Pad Software Inc., San Diego, CA, USA).

3 Results

3.1 Lymphocyte subsets

We found that the manipulation affected NK, but no other subpopulation of lymphocytes. Thus, no significant changes were observed in the proportions of T and B lymphocytes, or T helpers and T suppressors, and there was no change in the expression levels of the activation marker HLA-DR and KIR receptor CD158a in NK and T cells. In all individuals T cell frequency stayed constant (Figure 3A). T cell average levels on days 3 and 10 were 74.8 ± 4.5 and 75.5 ± 5.1 (not significant compared to initial 71.5 ± 4.5) (as % from initial levels 102.3% and 107.5%, Figure 4). In contrast, NK average level on day 10 was significantly decreased, 11.1 ± 4.1 compared to initial level 14.4 ± 3.7 (P=0.03, as % from initial levels 62.0%, Figure 4). NK average level on day 3 was 14.03 ± 4.70 (as % from initial levels 94.8%, not significantly different).

3.2 NK frequency and NKa

In contrast, the population of NK lymphocytes was very sensitive to manipulation (Figure 3B). The number of NK lymphocytes decreased in the majority of individuals (15/21) on day 10 after the manipulation (Figure 3B). Average NK levels were significantly decreased (Figure 4) although there were subjects, who showed opposite or no effect (Figure 3B). NKa also decreased in the majority of individuals after the manipulation (Figure 5); on the 3rd and 10th day after the manipulation, average NKa levels were significantly decreased, 43.9 ± 11.3 and 31.9 ± 8.9 compared to initial 52.0 ± 9.1 (P= 0.048 and 0.012 respectively) (Figure 4).

3.3 NKc

Simultaneous decreases in NK lymphocytes and NKa resulted in lower NKc. NKc levels were significantly decreased on days 3 and 10, and remained low through the final measurement at day 17 after manipulation (Figure 4). The observed effect varied among individual subjects, but in one-third of volunteers the decrease of NKc lasted for more than two weeks, and the rate of decline was similar to the effect observed when using intravenous immunoglobulin\textsuperscript{(14)} (Figure 6). Such cases fell into the common type of response and accounted for 39.1% (9/23) of individuals (Figure 6). Another 47.8% (11/23) of volunteers showed a slow response to the therapy or quick return to basal levels (Figure 6). Only three individuals (13%) did not show the therapeutic effect of the manipulation throughout the course of study (less than 20% from basal level) (Figure 6). In one case there was a slight increase in NKc on the 10th day.

The type of response may be individualistic. We showed this by repeating the manipulation in the same volunteer one-year after the first manipulation. The individual displayed “good response” in both cases (Figure 2). In 3 individuals from the weak or no response groups we...
4 Discussion

One of the important functions of NK lymphocytes is the elimination of unnecessary (apoptotic, hyper-activated) or dangerous (infected, potentially neoplastic) cells from the body\textsuperscript{[18,19]}. For this reason, NK lymphocytes are constantly ready for a cytotoxic reaction. In certain cases, this potential can accumulate excessively and become uncontrollable. It is possible that female patients with reproductive losses associated with high NKc have a similar imbalance in the NK function. That is, when the cytotoxic function begins to dominate over other physiological implantation-necessary functions of the NK system.

In our previous studies, we have demonstrated that the balanced function of NK lymphocytes is favorable for the implantation and carrying of a pregnancy. Similarly, there is an optimal range of values for NKa\textsuperscript{[12]}, expression of CD158a\textsuperscript{[20]} and expression of CD8\textsuperscript{[21]}; deviation from those ranges is adverse to reproductive function.

The CT has no pro-inflammatory effect, because it is devoid of damaging factors and pyrogenicity. Therefore, according to the “danger theory”, this impact would likely have a tolerogenic or suppressive effect instead of activating or pro-inflammatory effect\textsuperscript{[22]}. In this experiment, we confirmed this idea. In addition, the low cost of this method and its non-invasiveness makes it a very promising approach to reducing excess NKa in patients with reproductive losses. The individual variances of the effects are likely due to variance in dose administration of cupping. In future studies, we plan to improve the pattern of therapy to achieve more stable and predictable results.

5 Competing interests

All the authors have no conflicts of interest.

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