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Original Research

Wet Cupping Therapy Ameliorates the Inflammatory Responses in Mice Model of Allergic Asthma: An Experimental Histopathological Study

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Abstract

As an inflammatory disease of the lung, asthma is characterized by bronchoconstriction, mucus hypersecretion, inflammatory mediator release, and eosinophil recruitment. Cupping therapy is an ancient method of treatment for a vast range of ailments. This study aimed to evaluate the anti-asthmatic effects of wet cupping therapy (WCT) in a mouse model. A total number of 35 Balb/c mice were randomly divided into five groups (n = 7): Negative and positive control groups were administered Phosphate Buffered Saline (PBS) and ovalbumin (OVA), respectively. The remaining three OVA-challenged groups were treated with budesonide, one session, and two sessions of WCT. Finally, eosinophil counts, the gene expressions, and the protein levels of interleukins IL-5, -13, and -33 were measured in bronchoalveolar lavage fluid (BALF) of mice. Lung tissues were removed and kept for histopathological evaluations. Both eosinophil counts and interleukin levels in BALFs were significantly diminished following WCT. Moreover, WCT prevented hyperplastic growth of

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goblet cells, overproduction of mucus, and inflammation of peribronchial and perivascular areas of lung tissue of mice compared to positive control group. Interestingly, the anti-inflammatory effects of WCT against asthma were comparable to budesonide. Our data suggested that the anti-asthmatic effects of WCT were mediated by reducing eosinophil trafficking and modulating Th2 inflammatory cytokines, leading to the histological changes of the lung. This may propose WCT as an efficient therapeutic approach to mitigate inflammatory complications of asthma.

Keywords: Persian medicine; Wet cupping therapy; Asthma; Inflammatory response; Interleukins

Introduction

Asthma is an inflammatory disease affecting airways, which is characterized by the triad symptoms of wheezing, cough, and breath shortness. Bronchospasm and bronchoconstriction complications in asthma are occurred due to the trigger of inflammatory processes in the airways [1]. Currently, more than 330 million people around the world are suffering from asthma [2]. Moreover, it is responsible for more than 5000 deaths in the United States, annually [3]. Asthma occurs in two different forms, which include allergic and non-allergic phenotypes. There are two criteria for the differential diagnosis of allergic and non-allergic asthma in clinical settings, which are based on the presence or absence of allergic reaction and immunoglobulin E responses to some environmental allergens in vitro. Although some potential mechanisms have been demonstrated for allergic asthma, its exact mechanism remains to be elucidated [4]. Inflammatory responses of asthma in the initiation of the disease may be influenced by the predominance of eosinophil or neutrophil or both of them. The eosinophilic phenotype of asthma comprises a wider spectrum of the disease severity, ranging from mild-to-moderate to severe; however, the neutrophilic form of disease commonly shows a severe phenotype. Immune responses due to the eosinophilic form of asthma mainly occur through T helper 2 (Th2) cell-released cytokines, but Th17 cell activation is a dominant event in the initiation of neutrophilic asthma [5]. The most common form of asthma is known as allergic asthma, which is triggered by the initiation of inflammatory responses to environmental allergens [6]. The eosinophilic attack during allergic asthma triggers a chronic inflammatory condition in the airways and leads to damage to epithelial cells of the airways [7]. This can activate airway tissue remodeling leading to histopathological changes in the airways, which include hyperplastic growth of goblet cells, fibroblasts, and airway smooth muscle as well as the induction of angiogenesis and accumulation of extracellular matrix components. As a result, the disease enters a severe state by accelerating lung dysfunction and airway blockage [8].

Finding an efficient therapy for allergic asthma as the most prevalent chronic respiratory disease is a major challenge for public health. Allergic asthma is associated with some interleukins including IL-4, IL-5, and IL-13, which

are involved in mucus overproduction, airways hyperresponsiveness, airway eosinophilia, smooth muscle spasm, and immunoglobulin E (IgE) synthesis [9]. Therefore, the treatment of this disease is mainly based on the suppression of these immune cytokines. Inhaled corticosteroids are commonly used drugs for the treatment of asthma, however, their long-term use is accompanied by various adverse side effects including diabetes, osteoporosis, and respiratory infections [10]. Thus, it is a critical requirement to seek more effective treatments with fewer complications.

Persian medicine is a traditional and comprehensive medicinal system which is based on humoral medicine [11,12]. Cupping therapy is one of the therapeutic methods used in Persian medicine. This method is used in a wide range of diseases. It is generally divided into two types of wet cupping and dry cupping [13]. Dry cupping is usually performed by applying negative pressure sucking on the skin without any bloodletting [14]. In addition to venesection (*Fasd*) and leech therapy, wet cupping therapy (WCT) is one of the main bloodletting methods in Persian medicine. According to traditional Persian medicine, WCT is based on the creation of a short time suction on the skin to cause bloodletting after superficial scarification of the congested skin [15]. WCT has been shown to be an effective therapeutic approach for a variety of diseases such as low back pain [16], neck pain [17], carpal tunnel syndrome (CTS) [18], knee osteoarthritis [19], and herpes zoster [20]. It has also been used for the treatment of acute and chronic inflammation and immune system disorders. WCT decreases oxidative stress and modulates the release of inflammatory cytokines, resulting in the regulation immune system [21,22]. The present study aimed to assess the anti-asthmatic effects of WCT in an animal model of allergic asthma.

Methods

Reagents

The following materials were used in the present study: Budesonide (Ramopharmin, Iran), ovalbumin (OVA; Sigma-Aldrich, USA), Aluminum hydroxide (Sigma-Aldrich, USA), TRIzol (Invitrogen life technologies, USA), urethane (Sigma-Aldrich, USA), Mouse IL-5, IL-13 and IL-33 ELISA kits (Abcam, USA), and cDNA synthesis kit (Thermo Scientific, USA).

Animals

Thirty-five Balb/c female mice were purchased from Pasteur Institute, Tehran, Iran. The animals were 6-8 weeks-old weighing from 15-20 g at the time of treatments. All animals were housed under standard conditions defined in the guideline of the National Institutes of Health (NIH) for the use and care of laboratory animals. These conditions include 22 ± 1 °C temperature, a relative humidity of 50 ± 5%, a 12 h light/dark cycle, and an allergen-pathogen-free environment for one week prior to the sensitization method. During the experimental period, animals had limitless access to food and tap water. Moreover, the study protocol was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (No.: IR.SB-

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Experimental design

35 Balb/c mice were randomly divided into five groups (n = 7 for each group): the mice in group 1, as a negative control, were treated with an intraperitoneal injection of Phosphate Buffered Saline (PBS); Group 2 animals were challenged with an intraperitoneal injection of ovalbumin (OVA) as a positive control; Group 3 pretreated with OVA intraperitoneally, and subsequently received 1% budesonide orally; Group 4 mice were challenged with OVA, and then were treated with one session wet cupping on day 26; Group 5 was pretreated with OVA solution, and thereafter were treated with two sessions wet cupping on days 25 and 28. Details of the wet cupping method have been described in a previously published paper [23].

Asthma induction

The sensitization of animals and the induction of allergic asthma were performed during a month. Briefly, a solution comprising 50 µL aluminum hydroxide and 20 µg OVA dissolved in 50 µL PBS (pH 7.4) was prepared to sensitize all groups via intraperitoneal (i.p) injection of the solution on days 1 and 14 of the experiment. On days 24, 26, 28, and 30 of the experiment period, the animals were challenged with 30 min inhalation of OVA (1% in normal saline) utilizing an ultrasonic nebulizer apparatus (NB- 500, Rossmax, Switzerland). On day 31, the animals in each group were sorted into two unequal groups; the first group of 4 animals was anesthetized by urethane solution and their bronchoalveolar lavage fluid (BALF) was collected via tracheal cannulation and kept at freezer (-70°C) until further experiments. The second group of 3 animals was euthanized and their lung tissues were dissected and used for histopathological assessments [24].

Cytokine measurement and Eosinophil quantification

The BALF samples of mice were used to measure the protein concentrations of Interleukins (IL-5, IL-13, and IL-33) and quantify the number of eosinophil cells. Accordingly, the frozen BALF specimens were thawed, and subsequently, the levels of the mentioned interleukins were quantified by using an ELISA kit (Abcam, USA) according to the manufacturer's procedures. The number of eosinophil cells was counted in eight non-overlapping microscopic fields and averaged.

RNA extraction and real-time polymerase chain reaction

To determine the immunomodulatory effects of wet cupping on allergic asthma complications, the expression levels of IL- 5 and -13 genes in BALF specimens were measured by quantitative Real time-PCR method. Briefly, the total RNA was extracted from BALF cells using TRIzol solution. Afterward, the cDNAs were synthesized from the extracted mRNAs using a cDNA Synthesis Kit. The changes in the mRNA expression of IL-5 and -13 and GAPDH gene (as an internal control gene) were quantified by using quantitative reverse transcriptase PCR (qRT-PCR) in a rotor gene apparatus (Qiagen,

Hilden, Germany) with a SYBR GREEN® detection system [25]. To ensure reaction specificity and primer-dimer artifacts, post-amplification melting-curve analysis was performed. The sequences of primers that were utilized in this present investigation are shown in Table 1.

Table 1. Primer sequences used in this study

Gene name	Forward primer	Reverse primer
IL−5	5'- ATCCAGGAACTGCCTCGTC -3′	5'- ACATTGACCGCCAAAAAGAG -3′
IL−13	5'- AATAAGATCAAGAAGAAATGTGCTCAA -3′	5'- GGTCCACACAGGGCAACT -3′
GAPDH	5'-GGTCCTCAGTGTAGCCCAAG-3	5'-TGTTCCTACCCCCAATGTGT-3

IL, Interleukin

Histological evaluation

As described before, after euthanizing three animals in each group on day 31, their lung tissues were dissected, fixed by immersing them in 10% neutral buffered formalin, and embedding them in paraffin. Thereafter, the paraffin-embedded tissues were subsequently cross-sectioned by microtome and the tissue slides were stained by using the hematoxylin and eosin (H&E) method. Finally, the stained tissue sections were analyzed by an expert pathologist to evaluate and report any histopathological alterations [24].

Statistical analysis

The values presented in the present study are expressed as mean ± SD. One-way analysis of variance (ANOVA) followed by Newman-Keuls test was used to compare the data between test and control groups. Data were analyzed using SPSS software version 17.0. A p value of < 0.05 was considered a statistically significant level.

Results

Effect of WCT on eosinophil count in BALF samples

Figure 1 illustrates that the treatment of mice in the positive control group with OVA solution significantly incremented the eosinophils count in their BALF specimens compared with PBS-treated mice, as negative healthy controls ($P < 0.05$). Treatment of mice with budesonide remarkably declined the number of eosinophil cells of BALF samples in comparison to OVA-treated positive control group ($P < 0.05$). Interestingly, one and two sessions WCT more significantly lowered the eosinophil counts in BALF samples from the asthmatic group in comparison to OVA-administered mice. There were no significant differences in the number of eosinophils between one session and two session WCT groups.

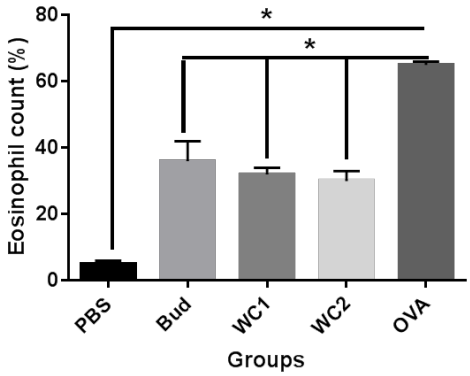

Figure 1. The alleviating effect of wet cupping (WC) therapy on eosinophil number in BALF samples of Ovalbumin (OVA)-induced asthmatic mice. The animals in negative control group were treated with Phosphate-buffered saline (PBS) and the positive control animals received OVA. WC and Budesonide (Bud)-treated animals were first pretreated with OVA to create asthma. The eosinophil counts in all groups are shown as mean ± SD of three replicate samples. $P < 0.05$ was considered statistically significant.

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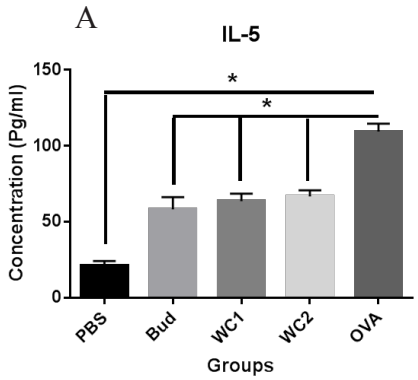
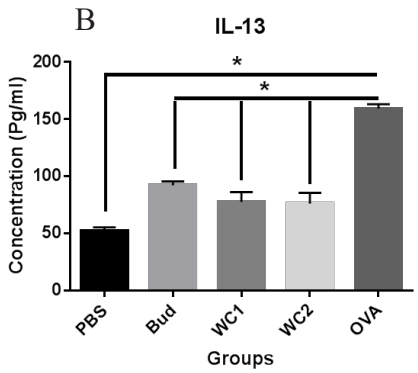
Effect of WCT on cytokine levels in BALF samples

As shown in Figures 2A, B, and C, the sensitization of mice with OVA in the positive control group significantly augmented the concentrations of IL-5, IL-13, and IL-33 in their BALFs as compared with the PBS-treated negative control group ($P < 0.05$). The treatment OVA-induced asthmatic group with budesonide markedly hindered the enhancement of IL-5, IL-13, and IL-33 in their BALF samples ($P < 0.05$). Moreover, using one and two sessions of the wet cupping method, we demonstrated that this therapeutic approach caused a significant reduction in the concentrations of IL-5 and IL-13 in the BALF samples of OVA-challenged animals, but had no remarkable effect on the concentrations of IL-33 in these two groups.





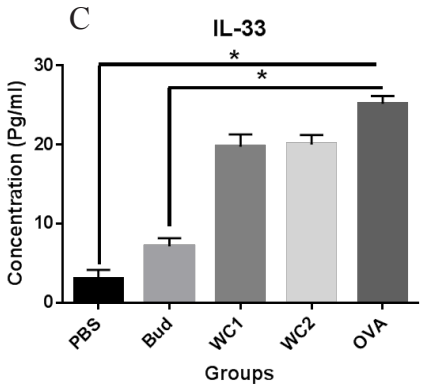


Figure 2. Effects of WC therapy on the BALF levels of IL-5 (A), IL-13 (B), and IL-33 in asthmatic mice (C). The mice in the OVA-sensitized group showed the increases in the levels all three interleukins in comparison to negative controls. Budesonide treatment significantly decreased the protein levels of these cytokines ($P < 0.05$). WC considerably suppressed IL-5 and IL-13, but didn't change IL-33 levels. All data are expressed as mean ± SD of three replicate specimens.

Effect of WCT on the gene expression of cytokines

For the estimation of the expression levels of IL-5 and 1L-13 in BALFs of all groups, the quantitative RT-PCR technique was used. The analysis of the mRNA expression of these two interleukins in BALF samples collected from OVA-induced asthmatic mice showed that OVA considerably augmented their mRNA expressions when compared with the PBS-treated group ($P < 0.05$). Nonetheless, their expression levels in BALFs of the budesonide-treated group were significantly low in comparison to OVA-sensitized animals ($P < 0.05$). Interestingly, the treatment of animals with one and two sessions of WCT more significantly ($P < 0.05$) hampered the increase in the mRNA expressions of both IL-5 and IL-13 (Figures 3A and B).

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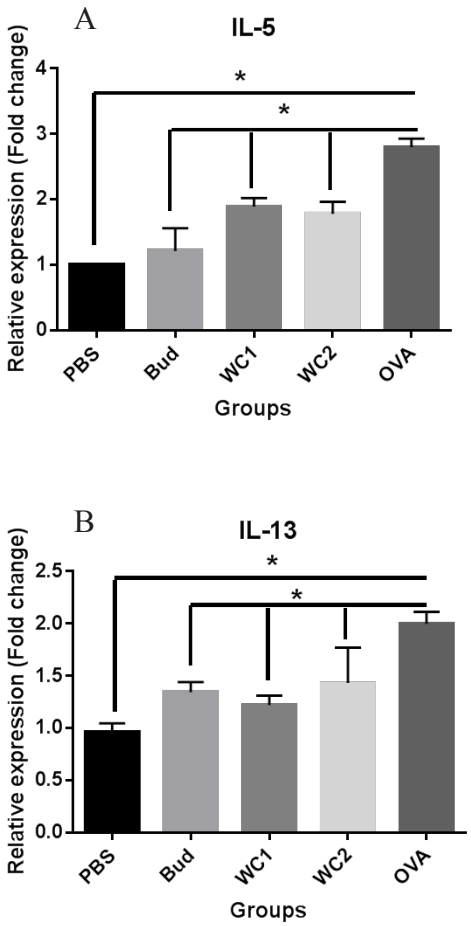

Figure 3. Downregulating impact of WC on the gene expression of IL-5 (A) and 1L-13 (B) in BALF samples. OVA challenge of positive control mice significantly enhanced the mRNA expressions of IL-5 and IL-13 in BALF samples as compared with PBS-treated group. Budesonide and WC therapy significantly declined the expressions of these cytokines. The experiments were done in triplicate and the results are expressed as mean ± SD and $P < 0.05$ was considered a statistical significant level.

Effects of WCT on the histopathology of lung tissue

Analysis of histological alterations of the lung tissues is shown in figure 4 and table 2. According to our analyses, the OVA-sensitized mice significantly enhanced in the infiltration and migration of inflammatory cells into perivascular and peribronchial areas of the lung tissue sections (Figure 4A). However, negative control mice had a normal histological appearance in their lung tissue with no observable inflammatory changes (Figure 4B). Therefore, the OVA challenge could accelerate the migration of infiltrates into the asthmatic lungs. Budesonide and WCT showed significant regression in the migration of the inflammatory cells into both peribronchial and perivascular tissues (Figures 4C, D, and E). Table 2 represents data of mucus secretion, goblet cell hyperplasia, and perivascular and peribronchial inflammation in different treatment groups. As shown in this table, asthma induction by OVA meaningfully enhanced the mentioned parameters in asthmatic lungs in comparison to negative control ones ($P < 0.05$). The data from the histological examination of budesonide-treated mice demonstrated a significant decline in the hyperplastic proliferation of goblet cells, hypersecretion of mucus, and inflammation in the peribronchial and perivascular areas of lungs when compared with OVA group. Interestingly, both one and two sessions of wet cupping remarkably inhibited the elevation of these parameters in comparison to OVA-treated mice ($P < 0.05$).

Table 2. Histological examination of lung tissues

	Goblet cells	Mucus (%)	Peribronchial inflammation	Perivascular inflammation
PBS	1±0.2	25±5	0.5±0.1	0.5±0.1
OVA	4±0.1*a	100±5*a	4±0.2*a	4±0.2*a
Bud	1.3±0.7*b	35±10*b	1.1±0.2*b	1.2±0.2*b

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WC1	2.5±0.5*b	25±15*b	1.5±0.1*b	1±0.7*b
WC2	2.5±0.2*b	25±5*b	1.5±0.2*b	1.5±0.5*b

OVA; Ovalbumin, PBS; Phosphate-buffered saline, Bud; Budesonide, WC1; Wet cupping (One session), WC2; Wet cupping (Two sessions). All data are presented as mean ± SD. *P < 0.05 was considered statistically significant: a) in comparison to PBS-treated group and b) in comparison to OVA-treated group.

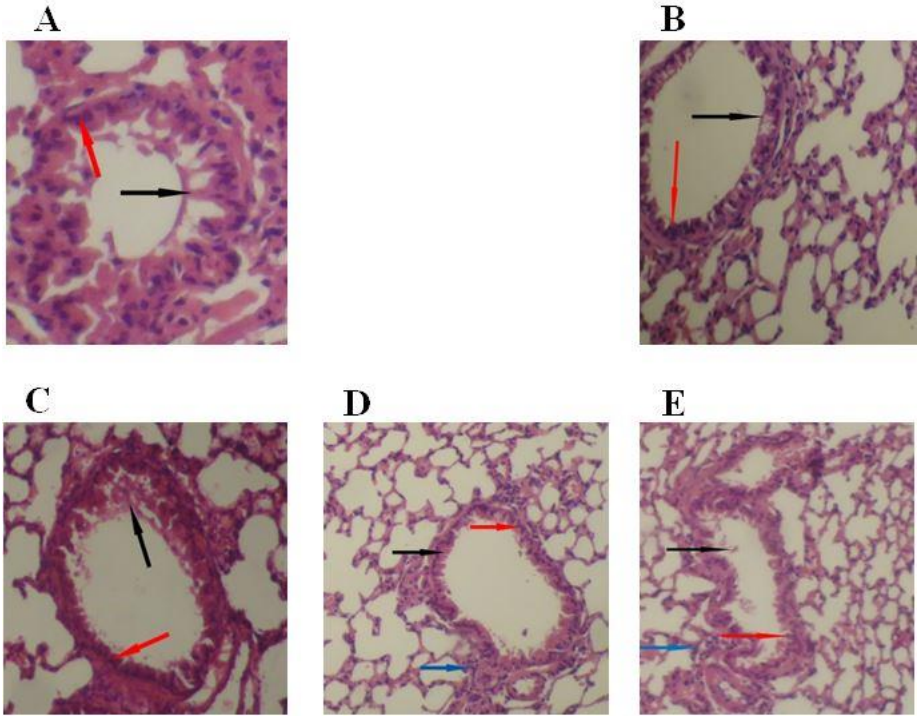

Figure 4. Histopathological examination of lung tissues stained with Hematoxylin-eosin (H&E). A) The positive control asthmatic group which was sensitized with OVA; B) The negative control group which was treated with PBS; C) The budesonide-treated asthmatic group; D) The tissue section obtained from asthmatic animals treated with one session WC; E) The asthmatic lungs treated with two sessions WC.

Discussion

The present investigation was conducted to reveal the immunomodulatory effects of WCT on inflammatory complications of OVA-induced allergic asthma in a murine model of the disease. We used one and two sessions of WCT to assess its therapeutic effect on asthma. Based on our observations, OVA sensitization of lungs and airways of Balb/c mice could induce asthmatic symptoms in mice by elevating the number of eosinophils in their collected BALF samples. However, both one and two sessions of the wet cupping method inversed this effect by decreasing the number of these immune cells in BALFs. The protective activities of WCT were compared with budesonide, as a standard drug for asthma treatment. Budesonide is an anti-inflammatory drug that has been effectively used as a clinical medication for the treatment of asthma [26]. According to our findings, the effect of wet cupping on eosinophil count was comparable to that of budesonide. Eosinophils play a key role in the inflammatory response of the lungs by inducing lung tissue remodeling and unfavorable inflammatory responses of the airways [27]. Thus, the accumulation of eosin-

ophils in the airways of the pulmonary system triggers a cascade of devastating events that exacerbate asthma symptoms [28,29]. These adverse side effects of the eosinophilic attack on the pulmonary system are due to the degranulation of these cells and the release of their toxic proteins into the lung tissue [30]. One of the major hallmarks of the severe form of asthma is the enhanced trafficking of eosinophilic cells. This increased number of eosinophils involves Th2 cells and type 2 innate lymphoid cells, which release Th2-related cytokines such as IL-4, IL-5, and IL-13 and trigger inflammatory processes [31,32]. These types of inflammatory cytokines are major role-players in the induction and promotion of type-2 inflammatory responses in asthma [33]. According to some reports, sensitization of epithelial cells lining the airways can also trigger these responses and induce the production and release of IL-25, IL-33 [33]. Our findings unraveled that the sensitization of Balb/c mice with OVA in the asthmatic group considerably raised the concentrations of IL-5, -13, and -33 in BALF samples of the animals. However, budesonide as well as one and two sessions of WCT significantly decreased the concentrations of the mentioned interleukins in BALFs. Analysis of data of Real time-PCR also affirmed that both budesonide and wet cupping therapies negatively regulated the gene expressions of IL-5 and IL-13 in comparison to OVA-sensitized mice. The pivotal roles of interleukins in triggering the inflammatory processes of various body organs have been numerously reported in the literature. The results of a study by Lee and colleagues showed that plasma cupping therapy, as a novel form of wet cupping method, efficiently hindered the activation of inflammatory responses through blacking the expression of pro-inflammatory interleukins such as IL-1β and IL-6, which was mediated by tumor necrosis factor-alpha (TNF-α)-dependent cascade [34]. Zhang, et al. also found that cupping therapy TNF-α and IL-6 expression and release in peritoneal fluids of a mouse model of cupping treatment method. They found that the inhibiting effect of cupping treatment on these pro-inflammatory cytokines is mediated by elevation of some anti-inflammatory lipids [35]. This may be a probable mechanism for the anti-inflammatory capacity of the cupping method in some body organs. Another possible mechanism for the anti-inflammatory ability of cupping therapy has been suggested to be exerted by the macrophage-mediated release of heme-derived substances such as carbon monoxide, biliverdin, bilirubin, and iron, which have anti-inflammatory activities and also motivate a shift of macrophages to an anti-inflammatory M2 phenotype [36]. One of the major roles of IL-5 is to recruit eosinophils and increase their trafficking and survival in different tissues [37]. IL-13 also acts by inducing the proliferation of eosinophils and stimulating airways and lungs to produce mucus [38]. IL-33 secretion into body fluids is also accompanied by the activation of a number of inflammatory cells such as eosinophils, basophils, mast cells, and macrophages causing inflammatory responses [39]. In looking at our data, the curative effects of WCT on ovalbumin-induced asthmatic complications may be related to its capacity in the suppression

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of mRNA expression and protein concentration of Th2-type interleukins. This may suggest a potential mechanism for the anti-inflammatory and anti-asthmatic impact of this therapeutic method.

As indicated in table 2, treating the mice with the wet cupping method, at the levels comparable to budesonide, prevented the goblet cell hyperplasia, mucus hypersecretion as well as peribronchial and perivascular inflammations compared with ovalbumin-treated mice. Further histopathological examinations also revealed prohibiting effects of WCT on the influx of immune cells into peribronchial and perivascular areas of the animal's lungs in comparison to ovalbumin-induced asthmatic mice. A study by Xing, et al. on the therapeutic effect of moving cupping therapy, a type of dry cupping, on psoriasis provided evidence to declare that the mentioned method can suppress inflammatory processes in psoriatic skin and prevent immense thickening of scars in this tissue [40]. However, there are some controversial ideas about the type of immunomodulatory function of WCT between scientists. For example, Soleimani, et al. by comparing the dominance of Th1 and Th2 responses between wet and dry cupping therapies in Persian medicine indicated that wet cupping enhances the number of Th2 type cells by measuring the gene expression of GATA-3, as a transcription factor of Th2 cells. However, they have not measured the concentrations of Th2 cytokines to give an appropriate picture for the Th-2 cell-produced inflammatory cytokines [41]. On the contrary, the results of the present study indicated that WCT significantly decreased Th-2 cell-produced cytokines at functional protein levels. Taken together, some previous studies imply that WCT improves natural immunity and represses pathological immunity by blocking the production of autoantibodies and inflammatory cytokines [42].

Conclusion

The results of the present investigation highlighted the anti-inflammatory and anti-asthmatic function of WCT on ovalbumin-induced allergic asthma in a mouse model. Based on the present data, it is suggested that the mentioned treatment exerts its protective effects on asthma complications by modulating eosinophil trafficking, Th2 cell-mediated responses, and histological alterations due to the induction of the inflammatory processes in lungs and airways. Moreover, our findings confirmed that wet cupping effects on the amelioration of the inflammatory complications of asthma were comparable to budesonide, as a standard anti-inflammatory drug. Therefore, the present findings may suggest WCT as a promising approach for the relief of inflammatory complications of asthma. However, while our results are in line with the expected trends, they should be interpreted with caution and do not be generalized to the human population as asthma is a multifactorial disorder with different variables.

Abbreviations

WCT, Wet cupping therapy; PBS, Phosphate Buffered Saline; OVA, Ovalbumin; Budesonide, Bud; BALF, Bronchoalveolar lavage fluid; Th2, T helper 2; IgE, Immunoglobulin E; CTS, Car-

pal tunnel syndrome; NIH, National Institutes of Health; IL, Interleukin; qRT-PCR, Quantitative reverse transcriptase PCR; TNF-α, Tumor necrosis factor-alpha.

Conflict of Interests

The authors declare that they have no conflict of interest.

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