Porcupine flesh homogenate induces T-bet and IFN-γ expression in mononuclear cells of asthmatic patients: possible molecular mechanism of a zoo-therapeutic treatment

Atieh Rafatmanesh1*, Younes Aftabi2, Saeid Abediankenari3, Mojtaba Najafi4 & Siavash Abedi5

1-3Immunogenetic Research Center, Immunology Faculty, Mazandaran University of Medical Science, Sari, Iran;
2Tuberculosis and Lung Research Center, Tabriz University of Medical Sciences, Tabriz, Iran;
4Department of Animal Sciences and Fisheries, Mazandaran Agriculture Sciences and Natural Resources University, Sari, Iran;
5Department of Internal Medicine, Faculty of Medicine, Mazandaran University of Medical Sciences, Sari, Iran

E-mail: Atiehrafatmanesh@gmail.com

Received 26 October 2016, revised 16 January 2017

The traditional use of animals and animal-derived products for medicinal purposes recently entitled as zoo-therapy. The porcupine (Hystrix spp.) belongs to a family of herbivorous rodent that lives in southern Europe and Asia. Traditionally, porcupine body parts have excessive medicinal values in endemic people zoo-therapeutic prescribes for treatment of illnesses such as gastritis problems, typhoid and asthma. Asthma is one of the most common chronic diseases worldwide that has characterized by reduction of T-helper type 1 cells and their produced IFN-γ cytokine because of T-bet transcription factor reduced activity. In this regard, current study aimed to investigate the molecular pathways of traditionally reported therapeutic effects of porcupine-flesh homogenate extract (PFHE) on asthma patients. After blood sampling of 26 asthmatic cases, peripheral blood mononuclear cells (PBMNCs) isolated, cultured and incubated with PFHE for 72 hrs. Then, T-bet mRNA expression and IFN-γ levels assessed using real-time PCR and ELISA methods respectively to find out if PFHE affects these factor levels. Results showed that PFHE significantly induced T-bet mRNA expression and elevates IFN-γ production in patients' cultured PBMNCs.

Keywords: Zoo-therapy, Porcupine, Asthma, T-bet, IFN-γ

IPC Int. Cl.: A61K, A61K 36/00, A01D 11/18, A01D 14/06, A01D 4/49, A01D 14/05

Since the ancient times, man has been using animals and plants for their essential needs, especially for food and medicine. Recently, utilization of animals and animal-derived products in medicine and treatment practices is defined as zoo-therapy. One of the valuable animals in zoo-therapy is Porcupine that belongs to Hystrix spp. It has excessive medicinal values in traditional zoo-therapy of some aborigines and its body parts have been used in the treatment of a variety of illnesses. For instance, in the India porcupine blood and the alimentary canal utilizes to treat breathing troubles and asthma. Also, porcupine family Hystrix indica resides in Iran and in some regions of the country such as villages of Azarbaijan and Mazandaran asthmatic patients drink porcupine blood and eat its meat in order to treatment of their illness. Also, treatment with plants is reported in traditional therapies for asthma. Associated with the propensity for allergic responses, asthma is one of the most common chronic diseases worldwide. It is characterized by airway hyper-responsiveness, edema, and increasing in mucus secretion, which needs more efficient treatments via targeting of molecular pathways. Cellular immunology studies showed that T helper type 2 (Th2) cells over-activation in parallel with a reduced T helper type 1 (Th1) cell activity leads to the development of allergy and the inflammation in airways. Furthermore, studies on animal models revealed that increasing in Th2 cytokine and decreasing in Th1 cytokine production worsened allergic airway inflammation. Therefore, the idea of enhancement of Th1 cytokine production and reducing Th2 cytokines for the purpose of inhibiting allergic responses proposed and evaluated by researchers. Also, it is shown that Th1 cells inhibit Th2-induced effectors through the production of IFN-γ and consequently reduce the promotion of allergic airway inflammation. The Th1 cell development from
CD4+ T cells occurs under the influence of a specific protein, T-bet, which is a member of the T-box family of transcription factors. In fact, T-bet is a master determinant of Th1 lineage and T-bet deficient models, profoundly represent the lack of Th1 immune responses. In addition, some potential treatments of asthma resulted in T-bet increasing and then it was suggested that elevating of T-bet levels may act as a protective way against asthma.

According to local reports about using of porcupine blood and flesh in traditional methods for asthma treatment in Iran, this study aimed to evaluate possible effects of porcupine-flesh homogenate extract (PFHE) on T-bet transcription factor expression and IFN-γ cytokine production in asthmatic patient’s peripheral blood mononuclear cells (PBMNCs).

Methodology

Patients and sample preparation
Totally 26 asthmatic patients selected from the allergy and asthma center of the Mazandaran university hospital and whom admitted to Tooba Medical Diagnosis Laboratory (Sari, Iran). The participant group composed of 10 men and 16 females with mean age 32.33±10.48 (Table 1). Firstly, the peripheral blood samples collected after consent confirm of the patients. Then, the PBMNCs isolated from heparinized blood by centrifugation on a Ficoll histopaque 1.077 (Lymphoprep, Norway).

Porcupine-flesh homogenate extract (PFHE) preparation
The animal Hystrix indica sacrificed under ethical and practical standards in the department of Animal Sciences and Fisheries of Mazandaran Agricultural Sciences and Natural Resources University and then the supernatant of flesh homogenate extract prepared. The flesh pieces treated with a mix of hyaluronidase collagenase and DNA as enzymes in sterilized microtubes. Micro-tubes held in 4 °C for 24 hrs and after that in -20 °C for 10 min. Then, we froze micro-tubes contents maintaining them in -70 °C for 10 min and after that we immediately transferred them to 37 °C conditions. Next, we put tubes in liquid nitrogen for 30 to 60 seconds and again melted the contents in 37 °C. This part performed twice and finally we centrifuged the micro-tubes (205×gm/10min) and then harvested the supernatant.

Cell culture and treatment
Cells from interphases collected and washed three times with RPMI-1640 medium (Gibco, USA). Then, cells counted and viability of them determined by trypan blue exclusion. After that, PBMNCs (5×10⁶) cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin (Biosera, UK) and a series of 200, 400 and 800 µg/dl of PFHE for 72 hrs after optimizing in vitro (37 °C and 5 % CO₂). Early microscopic analysis revealed that 400 µg/dl of the PFHE results in the best condition of cell growth and proliferation in culture medium, then tests continued with this concentration. Both case and control samples were cultured duplicate, with and without of the PFHE treatment.

Total RNA extraction
After 72 hrs, the cells collected of plate and centrifuged for 10 min at 300×gm. The supernatant completely separated by pipetting in order to using in cytokine assay procedure. Then, the cell pellet washed with phosphate buffered saline and total RNA isolated using the protocol of RNeasyPlus Mini Kit (Qiagen, Hilden, Germany). Extracted RNAs were eluted in 50µl RNase-free water and stored at -80 °C. The quality of RNA verified by electrophoresis on agarose gel and the concentration of each RNA sample measured in A260 using the Pico Drop 2000 instrument (Thermo Fischer scientific Inc, UK).

cDNA synthesis
We used the cDNA synthesis kit (Thermo scientific, USA) for complementary DNA (cDNA) synthesis according to the manufacturer’s instruction. The steps include: incubation in 65 °C for 5 min, then, for 5 min at 25 °C that followed by 60 min incubation

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Case (Value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>Mean, years 32.33±10.48 range, years 14-51</td>
</tr>
<tr>
<td>Sex</td>
<td>Male 10 (38.5%) Female 16 (61.5%)</td>
</tr>
<tr>
<td>BMI</td>
<td>Male 25.84±4.84 Female 25.32±4.09</td>
</tr>
<tr>
<td>Blood groups</td>
<td>O+ 30.76% B+ 30.76% AB+ 15.38% A+ 23.07% O- Negative</td>
</tr>
<tr>
<td>Percentage of eosinophil</td>
<td>12.64±6.65</td>
</tr>
<tr>
<td>History of allergic asthma (year)</td>
<td>5.93±3.39</td>
</tr>
<tr>
<td>History of diabetes</td>
<td>Negative</td>
</tr>
<tr>
<td>History of medication consumption</td>
<td>Negative</td>
</tr>
<tr>
<td>Place of residence</td>
<td>County 19(70%) Village 7(30%)</td>
</tr>
</tbody>
</table>
at 42 ºC. Evaluation of concentration and purity of cDNA performed using Pico Drop 2000.

Real-time PCR
Assessing of T-bet mRNA expression performed using specific primers with Quanti Fast SYBR Green PCR Master Mix (Thermo scientific, USA) and elongation factor-1 (EF-1) gene expression used as a reference (Table 2). Real-time PCR run on an IQ5 RT-PCR Detection System (Bio-Rad, USA) and performed in a 20 µl tube that contained 10 µl of QuantiTect SYBR Green PCR master mix (Thermo scientific, USA), 1µl of forward and reverse primers (Bioneer, South Korea) and 1µL of first strand cDNA. Amplification was conducted using the following settings: stage 1: 5 min heating at 95 ºC, stage 2: 20 sec at 95 ºC, 30 sec at 60 ºC and 30 sec at 72 ºC (45 cycles). All measurements performed at least duplicate.

Measurement of IFN-γ cytokine
Levels of IFN-γ in PBMNCs culture supernatant fluid measured by enzyme immunoassays according to the manufacturer’s protocol (Boster, USA).

Statistical analyses
Statistical analysis carried out using SPSSver. 22.0. Data analyzed using non-parametric procedures to assess differences of T-bet expression and INF-γ levels with and without PFHE treatment in cultured PBMNCs. A value of \( p < 0.05 \) was considered statistically significant.

Results
T-bet mRNA expression
As shown in Fig. 1, T-bet mRNA expression in cultured PBMNCs of asthmatic patients increased after PFHE addition in cell culture significantly (\( p < 0.05 \)).

IFN-γ Levels
The 400 µg/dl of PFHE significantly increased levels of IFN-γ in cultured PBMNCs of allergic asthma patients (\( p < 0.05 \)) as shown in Fig.2.

Table 2—Sequences of the real time PCR primer sets

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Sequences</th>
<th>Product length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EF-1</td>
<td>Forward</td>
<td>CTGAACCATCCAGGCCAAAT</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCGTGTGGCAATCCAAT</td>
<td></td>
</tr>
<tr>
<td>T-bet</td>
<td>Forward</td>
<td>GATGCAGCGAGGAGTTTCAT</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCACAATCATCTGGGTACATT</td>
<td></td>
</tr>
</tbody>
</table>

Discussion
Adapted to the local people and conditions, the zoo-therapy uses traditional drugs of animal origin in the environment for the preparation of curative, protective and preventive medicinal substances. Indeed, the traditional medicine of China, India, Latin America and Brazil, Saudi Arabia and Jordan, Africa and Eurasia, Iran, Iraq and other middle-East countries, has a body of indigenous knowledge enriched by zoo-therapeutic approaches. Since ancient times ingredients of wild plants and animals’ by-products have been used in

Fig. 1—T-bet mRNA expression in cultured PBMNCs of asthmatic patients increased after PFHE addition in cell culture significantly (\( p < 0.05 \)). PFHE: Porcupine-flesh homogenate extract; PBMNCs: peripheral blood mononuclear cells.

Fig. 2—The 400 µg/dl of PFHE significantly increased levels of IFN-γ in cultured PBMNCs of allergic asthma patients (\( p < 0.05 \)). PFHE: Porcupine-flesh homogenate extract; PBMNCs: peripheral blood mononuclear cells.
directs activation of IFN-γ and ectopic expression of T-bet in murine Th2 cells. Expression is restricted to Th1 cells. The Protein T-bet is a member of the T-box family of transcription factors and its development is T-bet. The Protein T-bet is a transcription factor that manages this especial role in various effector cells such as Th1 subtypes. The main cells encounter specific antigens differentiate into cytokine, IFN-γ, respectively in Th2 and Th1 cells. Then, levels of Th1 cytokine, IFN-γ, and Th2 cell cytokine, IL4, could affect airway inflammation. It was suggested that T-bet, the main determinant of Th1 cell development, might protect against asthma by conducting Th1 cell proliferation that leads to more INF-γ production. Indeed, T-bet deficient mice exhibited a profound lack of Th1 immune responses and ectopic expression of T-bet in murine Th2 cells directs activation of IFN-γ. Also, a long-lasting asthma study revealed that T-bet deficiency is correlated with the increase of IgE that plays a crucial role in inflammatory events. In addition, polymorphism studies revealed that genetic variation at the T-bet locus confers susceptibility to asthma, airway hyper-responsiveness, and atopy.

The goal of asthma therapy is generally inhibiting of inflammation in the allergic airway. Pursuing this purpose many studies performed to discover how natural anti-inflammatory substances act and cause effects. For instance, studies on using plant derived anti-inflammatory materials such as D-pinitol and Quercetin in asthma treatment have shown that they increase T-bet expression and suppress GATA-3 transcription factor. The expression of GATA-3 is markedly up-regulated in cells differentiating along the Th2 lineage and conversely is down-regulated in cells differentiating in the Th1 pathway. Also, it is discovered that D-pinitol and Quercetin reduce allergic airway inflammation and hyper-responsiveness due to the alteration of Th1/Th2 polarization via the suppression of GATA-3 and increase of T-bet expression in asthma model mice. In addition, D-pinitol and Quercetin reduce the increased levels of IL-4, Th2 cytokine, and increase production of IFN-γ, Th1 cytokines, in asthma model and challenged mice. Similarly, our results showed that both in control and patient groups, T-bet expression increased after addition of PFHE in PBMCs culture significantly (p < 0.05). It seems that porcupine flesh homogenate contains substance(s) that act like plant-derived D-pinitol and Quercetin in increasing T-bet transcription factor and inducing IFN-γ production. In fact, T-bet controls the expression of the IFN-γ and its expression correlates with IFN-γ expression in Th1 cells. Thus, ectopic expression of T-bet transactivates the IFN-γ gene and induces endogenous IFN-γ production. Our results revealed that along increasing of T-bet expression in cell culture after PFHE treatment, IFN-γ levels increased also significantly (p < 0.05). It is shown that IFN-γ levels, reduce in allergic asthma patients. Taken together, our data showed that zoo-therapeutic treatment of asthma by utilizing PFHE may act via inducing expression of T-bet, Th1 cells specific transcription factor, and consequently raising of IFN-γ production.

Finally, it is very important to authors to declare clearly that it is just a descriptive study and we are extremely against to misbehavior with wildlife and we...
do not appreciate any injurious activities that probably harm animals’ life and existence especially under the pretext of medicinal intentions.

Acknowledgment
The authors would like to thank the management of Mazandaran University of medical science for providing the facilities and encouragement to carry out this work. The authors declare that no conflict of interest exists.

References
21. Gounni A, Aloufi A, Biswa R & Das AP, Zootherapeutic uses of animals by the Ao tribe of North-western region of the Kingdom of Saudi Arabia and


41 Chakravorty J, Meyer-Rochow VB & Ghosh S, Vertebrates used for medicinal purposes by members of the Nyishi and Galo tribes in Arunachal Pradesh (North-East India), *J Ethnobiol Ethnomed.*, 7 (2011) 1746.


