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

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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-γcytokine 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
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Since the ancient times, man has been using animals and plants for their essential needs, especially for food and medicine[1]. Recently, utilization of animals and animal-derived products in medicine and treatment practices is defined as zoo-therapy[2]. 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[3-5]. For instance, in the India porcupine blood and the alimentary canal utilizes to treat breathing troubles and asthma[6-8]. Also, porcupine family *Hystrix indica* resides in Iran[9] 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[10]. 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[11]. 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[12]. 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[13-15]. 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[16]. The Th1 cell development from

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$CD4^+$T cells occurs under the influence of a specific protein, T-bet, which is a member of the T-box family of transcription factors[17]. In fact, T-bet is a master determinant of Th1 lineage and T-bet deficient models, profoundly represent the lack of Th1 immune responses[18-20]. 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[18,19,21-24].

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 (Table1). Firstly, the peripheral blood samples collected after consent confirm of the patients. Then, the PBMNCs isolated from heparinized blood by centrifugation on a Ficollhistipaque 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 micro-tubes. Micro-tubes held in 4 ºC for 24 hrs and after that in -20 °C for 10 min. Then, we froze micro-tubes contents maintaining them in -70 ºC for 10 min and after that we immediately transferred them to 37 ºC conditions. Next, we put tubes in liquid nitrogen for 30 to 60 seconds and again melted the contents in 37 ºC. This part performed twice and finally we centrifuged the micro-tubes (205×gm/10min) and then harvested the supernatant.

Table 1—Description of patients with allergic asthma

Characteristics			Case (Value)
Age	Mean, years		32.33±10.48
	range, years		14-51
Sex	Male		10 (38.5%)
	Female		16 (61.5%)
BMI	Male		25.84±4.84
	Female		25.32±4.09
Blood groups	O^+		30.76%
	B^+		30.76%
	AB^+		15.38%
	A^+		23.07%
	O^-		Negative
Percentage of eosinophil			12.64±6.65
history of allergic asthma (year)			5.93±3.39
History of diabetes			Negative
History of medication consumption			Negative
Place of residence		County	19(70%)
		Village	7(30%)

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 (5×10^6) 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 % CO_2). 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 ºC for 5 min, then, for 5 min at 25 ºC that followed by 60 min incubation

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 °C, stage 2: 20 sec at 95 °C, 30 sec at 60 °C 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

Gene	Primers	Sequences	Product length (bp)
EF-1	Forward	CTGAACCATCCAGGCCAAAT	59
	Reverse	GCCGTGTGGCAATCCAAT	
T-bet	Forward	GATGCGCCAGGAAGTTTCAT	83
	Reverse	GCACAATCATCTGGGTCACATT	

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[25-27]. Indeed, the traditional medicine of China[28], USA[29], India[2,30], Latin America and Brazil[31,32], Saudi Arabia and Jordan[33], Africa and Eurasia[34], Iran, Iraq and other middle-East countries[35,36], 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

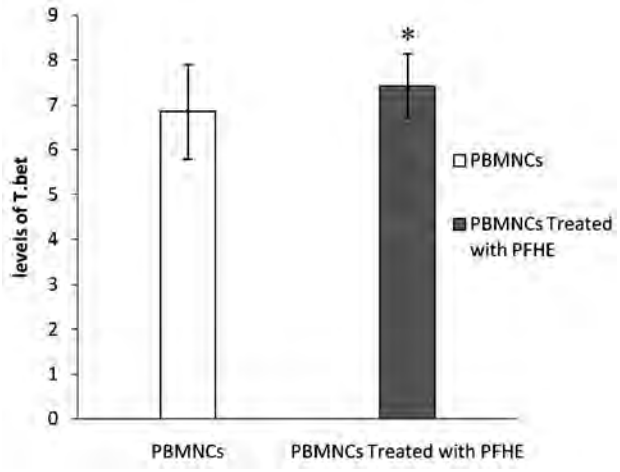

Fig. 1—T-bet mRNA expression in cultured PBMNCs of asthmatic patients increased after PFHE addition in cell culture significantly ($p < 0.05$).PFHE: Porcupine-flesh homogenate extract; PBMNCs: peripheral blood mononuclear cells.

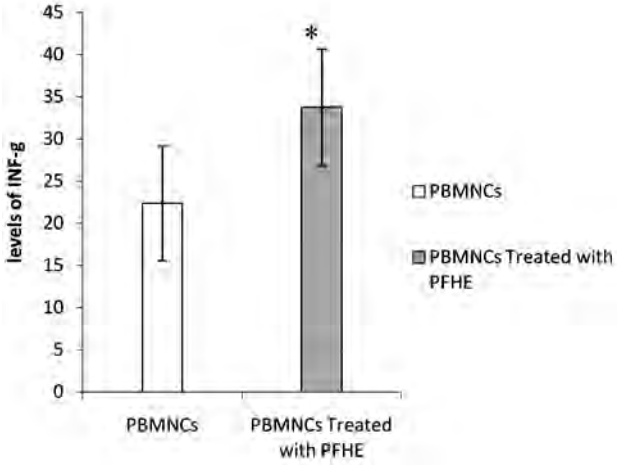

Fig. 2—The 400 µg/dl of PFHE significantly increased levels of IFN-γ in cultured PBMNCs of allergic asthma patients ($p < 0.05$). PFHE: Porcupine-flesh homogenate extract; PBMNCs: peripheral blood mononuclear cells

traditional medicine and presently they are considered an important source for providing raw materials in the preparation of modern pharmaceutics[25,37]. In fact, it is reported that more than half of the world's modern drugs are of biological sources[38,39]. Merriam-Webster's Collegiate Dictionary (2005 edition) defines Porcupine as: "any of various relatively large, slow-moving chiefly herbivorous rodents having sharp erectile spines mingled with the hair and constituting an old world terrestrial family (*Hystricidae*) and a new world chiefly arboreal family (Erethizontidae)". The *Hystrix indica* family of Porcupine lives in Iran[9] and has an important position in rural and traditional medicine. Native experts of zoo-therapy believe that the porcupine has excessive medicinal values[3] and they use its body parts in the treatment of burns, wounds, cuts, gastritis problems, typhoid and fever and asthma illnesses. Therefore, porcupine preserved parts, bile juice, fat, intestine and cooked or dried flesh and spine powder have been used as raw materials for treatment of various diseases in zoo-therapeutic practices[5-8,40,41]. Specifically, traditional medicine inventory of zoo-therapeutic practices prescribe porcupine blood and boiled alimentary canal as a remedy for asthma and other respiratory problems[6-8,35].

In the case of immune responses and inflammatory diseases such as asthma, it is well known that $CD4^+$ T cells have essential regulatory roles[17]. When $CD4^+$ T cells encounter specific antigens differentiate into various effector cells such as Th1 subtypes. The main transcription factor that manages this especial development is T-bet. The Protein T-bet is a member of the T-box family of transcription factors and its expression is restricted to Th1 cells[19,20,42,43]. Also, it is discovered that initial Th1 differentiation is dependent on IFNγ-induced T-bet expression[44].

Moreover, it has been shown using animal models that allergic airway inflammation is impaired by increased and decreased cytokine production respectively in Th2 and Th1 cells. Then, levels of Th1 cell 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[21,23,45]. Indeed, T-bet deficient mice exhibited a profound lack of Th1 immune responses[13] and ectopic expression of T-bet in murine Th2 cells directs activation of IFN-γ[13,43]. 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[46]. In addition, polymorphism studies revealed that genetic variation at the T-bet locus confers susceptibility to asthma, airway hyper-responsiveness, and atopy[47].

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[48,49]. 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[50]. 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[48,49]. Similarly, our results showed that both in control and patient groups, T-bet expression increased after addition of PFHE in PBMNCs 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[51,52]. 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[13-15]. Taken together, our data showed that zoo-therapeutic treatment of asthma by utilizing PFHE may act via inducing expression of T-bet, Th1cells 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.

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