Efficacy of Setarud (IMOD™), a novel electromagnetically-treated multi-herbal compound, in mouse immunogenic type-1 diabetes

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Abstract

Introduction: The aim of this study was to evaluate the effects and mechanisms of Setarud (IMOD™) as a multi-herbal medicinal formula on a mouse model of type 1 diabetes.

Material and methods: Autoimmune diabetes was induced by multiple low-dose intraperitoneal injection of 40 mg/kg of streptozotocin (STZ) for five consecutive days. IMOD™ was administered at an effective dose of 20 mg/kg/day for 21 days. After 21 days of treatment, the pancreases of the animals were separated and homogenized. In the pancreas tissue, the level of lipid peroxidation as thiobarbituric acid reactive substances (TBARS), total antioxidant power as ferric reducing ability of pancreas (FRAP), myeloperoxidase (MPO), and the concentrations of interleukin-1 (IL-1β) and tumour necrosis factor-α (TNF-α) were evaluated. Glucose changes were tested in the blood. Microscopic changes in the pancreas were followed by histological examinations.

Results: No significant difference was found between IMOD™ and diabetic control groups in blood glucose pattern. STZ-exposed mice showed a significant increase in pancreatic TBARS, MPO, IL-1β, and TNF-α levels, along with a significant decrease in FRAP value. Co-administration of IMOD™ significantly improved all the mentioned parameters disrupted by STZ administration except for blood glucose and histological changes.

Conclusions: IMOD™ could ameliorate oxidative and immunological distresses of type-1 immunogenic diabetes but could not normalize blood glucose. Further studies are recommended to clarify the effects of IMOD™ on immunological factors to address whether this new agent could be applied in diabetes prevention or therapy.

Key words: IMOD™, multiple low dose streptozotocin, diabetes, insulitis, antioxidant, oxidative stress.

Introduction

Type 1 diabetes is an autoimmune disease characterized by infiltration of lymphocytes or macrophages into the pancreatic islets and destruction of β cells leading to hyperglycaemia. Environmental triggers such as viruses or toxins in a genetically susceptible body may commence a series of

reactions and initiate an immune system response. T cell lymphocytes play a major role in beginning such a harmful process. Cytotoxic cytokines such as tumour necrosis factor α (TNF-α) and interleukin 1 (IL-1β) which are released from activated T cells result in inflammation within the pancreatic islets, called insulitis [1]. Following insulitis, free radicals are induced that accelerate the type 1 diabetes [2, 3].

Beside non-obese diabetic (NOD) mice and bio-breeding (BB) rats, multiple low dose (MLD) administration of streptozotocin (STZ) to mice is used as a model of type 1 diabetes in animals. Five days' consecutive low dose injection of STZ to mice starts an inflammatory response leading to insulitis resulting in type 1 diabetes [4].

In management of type 1 diabetes, immuno-modulators such as azathioprine, cyclosporine, nicotinamide, and new anti-cytokines or cytokine inhibitors, have been successfully applied to diminish lymphocytic infiltration to β cells and production of pro-inflammatory TNF-α and IL-1β, and the immune-mediated destruction of β-cells [5].

Previous studies have shown that some antioxidants and radical scavenging agents could counteract STZ-induced diabetes [6-11]. Preventive effects of such therapies are explained by the role of oxidative stress in pathophysiology of insulitis. Furthermore, β cells have been shown to contain fewer antioxidant enzymes including catalase, glutathione peroxidase and superoxide dismutase in comparison with other tissues which worsen the destructive effect of oxidative stress [12].

IMOD™ has been patented with the code of WO/2007/087825 for its immunomodulatory and anti-TNF-α capacities [13]. It is a three-component herbal extract treated with chemical trace elements and a special electromagnetic field. It was invented briefly by preparing ethanolic herbal extract from *Rosa canina*, *Urtica dioica*, and *Tanacetum vulgare*, adding selenium and urea and having been exposed to a pulsed electromagnetic field. The mechanism implicated for the immunomodulating effect of IMOD™ is increasing the amount of CD4 T-lymphocytes and strong antioxidative and anti-TNF-α potential. In addition, toxicology tests have demonstrated the safety of IMOD™ [14].

In the present study the effect of IMOD™ as a novel natural immunomodulator with very impressive antioxidative properties has been tested in MLD STZ-induced mouse type 1 immunogenic diabetes.

Materials and methods

Animals and Reagents

Eight to ten week old, male BALB/C mice (30-40 g) were taken from the animal house of the institute. They were kept in groups of 6 mice/cage. All animals received care in accordance with the national health guidelines and the design of the study was approved by the animal experiments ethics committee of Tehran University of Medical Science. IMOD™ was obtained from Pars Roos Co. (Tehran). Blood glucose was measured using an ACCU-CHEK glucometer (USA). Mouse TNF-α and IL-1β ELISA kits were purchased from Nima-Pouyesh Co. (Tehran).

Materials for measurement of thiobarbituric acid reactive substances (TBARS), total antioxidant power (ferric reducing ability of pancreas; FRAP), myeloperoxidase (MPO), protein, and sample preparation for histological assay were purchased from Merck Chemical Co. (Tehran). Protease inhibitor and STZ were obtained from Sigma-Aldrich (UK).

Treatment groups

Animals were divided randomly into four treatment groups. Diabetes was induced in mice by injection of STZ (40 mg/kg dissolved in citrate buffer, pH 4.5) intraperitoneally for 5 consecutive days in the second and fourth groups. Diabetic mice in D-IM and D-C groups were treated every day with an effective dose of IMOD™ (20 mg/kg, IP) and vehicle (normal saline) respectively for 21 days. Animals receiving IMOD™ treatment were injected with STZ 30 min prior to IMOD™. The other two groups (ND-C and ND-IM) received either normal saline or IMOD™ intraperitoneally for 21 days. Blood glucose was measured on days 1, 7, 14 and 21 from the tail vein. Insulin and C-peptide were not measured because the blood sugar is a good presenter of insulin status inside the murine body. Hyperglycaemia was defined as a non-fasting blood glucose level ≥ 11 mmol/l (200 mg/dl). Animals were sacrificed on day 21 and pancreas samples were removed for histological, biochemical, and cytokine assays.

Determination of pancreatic MPO, TBARS, FRAP, and cytokines

MPO assay

Pancreas biopsy was weighed before being placed into phosphate buffer (pH = 7) containing a protease inhibitor (PMSF), to achieve 100 mg/ml concentration of tissue. Then it was homogenized and centrifuged at 45 000 × g for 30 min. The supernatant was removed and frozen at –80°C until assay. To the sediment was added 10 ml of 50 mM phosphate buffer (pH = 6) containing 0.5% hexadecyl trimethyl ammonium bromide (HETAB) and 10 mM EDTA, sonicated and centrifuged at 12 000 × g for 20 min. Finally, the supernatant was removed and frozen at –80°C for MPO assay. During analysis, 100 µl of preserved supernatant was

mixed with 2.9 ml phosphate buffer containing 0.167 mg/ml O-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. MPO activity was measured spectrophotometrically as the change in absorbance at 460 nm. Results are presented as milliunits per mg protein [15].

TBARS assay

TBARS of the pancreas was determined from the preserved supernatant as mentioned above. Of the supernatant, 0.5 ml was added to 2.5 ml trichloroacetic acid (20%); after 10 min at room temperature, the sample was centrifuged (10 000 × g, 10 min), and the sediment was added to a reaction mixture consisting of 2.5 ml sulfuric acid 0.05 M, 3 ml of TBA 0.2%, and sodium sulfate 2 M. The mixture was incubated at 100°C for 30 min. After cooling to room temperature, 3 ml of n-butanol was added to the sample and centrifuged (10 000 × g, 10 min) and then the absorbance was measured at 532 nm. Results are expressed as nmol/mg protein [15].

FRAP assay

Of the above-mentioned preserved supernatant, 0.1 ml was added to a reaction mixture consisting of 2.5 ml of acetate buffer (300 mmol/l, pH = 3.6), 0.25 ml of ferric chloride (20 mmol/l), 0.25 ml of TPTZ (2,4,6-tripyridyl-s-triazine 10 mmol/l into hydrochloric acid 40 mM), then it was incubated for 10 min at 37°C. The absorbance was measured at 593 nm. Results are expressed as µmol/mg protein [15].

Protein assay

Total protein in pancreas samples was analysed using the Bradford protein assay. Briefly, diluted samples were mixed with Bradford reagent dye and after 5 min the absorbance was measured at 595 nm by the spectrophotometer. Albumin was used as standard.

Cytokines assay

The concentration of IL-1β and TNF-α in the preserved supernatant of the pancreas (described above) was determined using specific mouse ELISA kits. According to the procedure, a coloured product is formed in proportion to the amount of cytokine present in samples, and after adding stop solution to terminate the reaction, absorbance was measured at 450 nm as the primary wavelength and 620 nm as the reference wavelength. TNF-α and IL-1β levels were expressed as pg/µg protein. Details of the procedure have been described previously [15].

Histological assay

To evaluate the severity of insulitis at day 21, a portion of pancreas was fixed in 10% formaldehyde, embedded in paraffin, sectioned at 4.5 µm, and stained with haematoxylin-eosin. Insulitis was graded 0 to 3, according to infiltration of islets with lymphocytes as none = 0, less than 10% = 1, 10 to 50% = 2, and more than 50% = 3 [16].

Statistical analyses

Statistical analyses of the data were performed using StatsDirect statistical software version 2.7.2 (9/6/2008). One way ANOVA was followed by post-hoc Newman-Keuls multiple comparisons test. Kruskal-Wallis test was used to compare histological scores. A p value of < 0.05 was regarded as significant.

Results

Effect of IMOD™ on blood glucose

After injection of STZ, blood glucose in both treated groups was raised. Though blood glucose did not attain the diabetic range (more than 200 mg/dl) after 7 days, all animals in both STZ-treated groups were diabetic when tested after 14 days. As shown in Figure 1, there was no difference between diabetic animals given IMOD™ and diabetic controls in blood glucose pattern. Also there was no significant difference in blood glucose between the two non-diabetic (IMOD™ alone and control) groups. IMOD™ treatment had no significant effect on blood glucose changes (Figure 1).

Effect of IMOD™ on pancreatic oxidative stress

Following administration of IMOD™, oxidative stress indicators including MPO activity, FRAP, and TBARS were measured in pancreatic specimens of animals at the end of the study (day 21). Figure 2A shows MPO activity between groups. There was

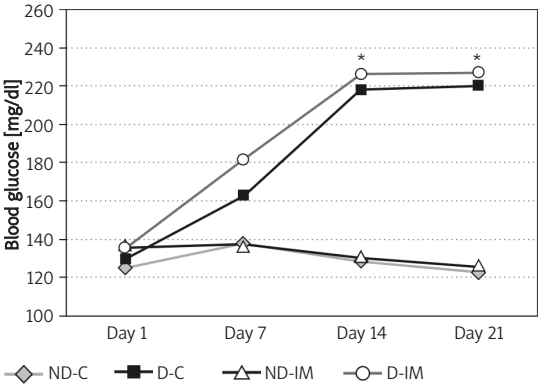

Figure 1. Non-fasting blood glucose changes in experimental groups. Difference in glucose value on days 7, 14, and 21 in D-IM and D-C compared with those of ND-C and ND-IM is significant at $p < 0.01$ (*). There was no difference between the diabetic group given IMOD™ and diabetic controls in blood glucose pattern

a significant difference between diabetic and other groups in MPO activity ($p < 0.05$). There was a significant decrease of MPO activity in IMOD™ -treated diabetic mice compared to the diabetic control group ($p < 0.05$).

Data of TBARS used to quantify lipid peroxidation in the mice pancreases are seen in Figure 2B. TBARS was elevated in STZ-treated (diabetic control) mice compared to other groups ($p < 0.05$). IMOD™ treatment reduced TBARS in the pancreas of both diabetic and non-diabetic mice ($p < 0.05$).

Data of changes in FRAP used to assess total antioxidant power are shown in Figure 2C. There was increased FRAP among IMOD™-treated animals. Total antioxidant power was the least in the diabetic control group ($p < 0.05$) while it was the greatest in both IMOD™-treated groups as compared with non-diabetic mice ($p < 0.05$).

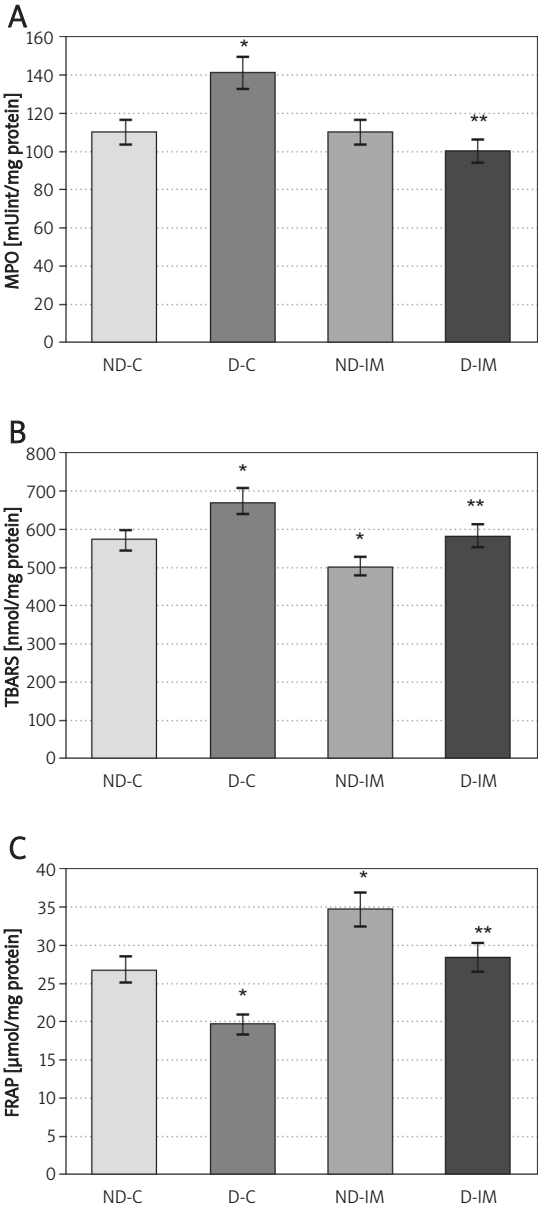

Figure 2. Pancreatic level of oxidative stress markers in experimental groups on day 21. IMOD™ (20 mg/kg/d, IP) decreased MPO activity (**A**) and lipid peroxidation, assessed by TBARS concentration (**B**), in pancreas samples of treated mice. Total antioxidant power (FRAP) of pancreatic tissue increased in IMOD™ treated groups (**C**)
**p < 0.01 comparing with ND-C; **p < 0.01 comparing with D-C*

Effect of IMOD™ on pancreatic cytokines

Two Th1 cytokines, IL-1β and TNF-α, were assessed at the end of the study in the pancreas sample of mice. Results are shown in Figure 3. As seen, there are significant differences in concentration of both cytokines in diabetic mice. IMODTM could lead to lower production of IL-1β and TNF-α in pancreas of treated mice.

Histological changes

Histological insulitis scores were higher in the pancreatic samples of diabetic mice than those of the controls on day 21 ($p < 0.05$). Insulitis index (Figure 4) in the IMOD™-treated group was not significantly lower than diabetic control mice.

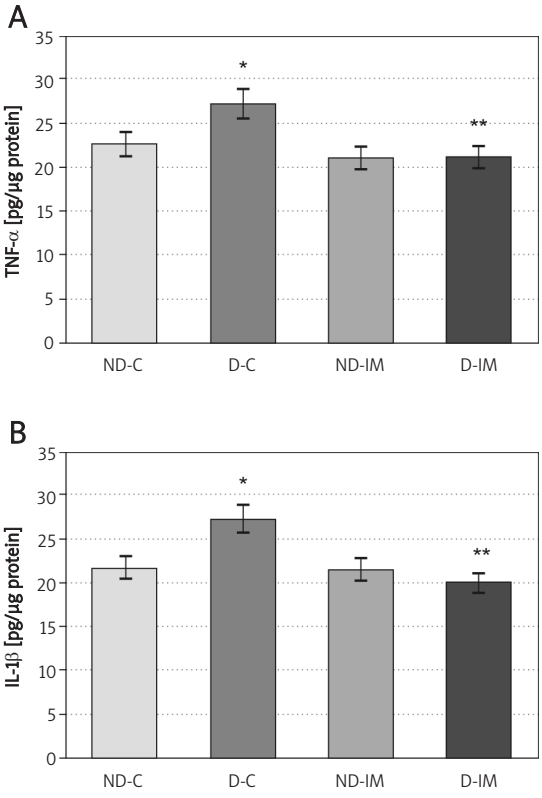

Figure 3. Pancreatic level of Th1 cytokines TNF-α and IL-1β in experimental groups on day 21. IMOD™ (20 mg/kg/d, IP) prevented increase in TNF-α (**A**) and IL-1β (**B**) in pancreas sample of treated mice
**p < 0.01 comparing with ND-C; **p < 0.01 comparing with D-C*

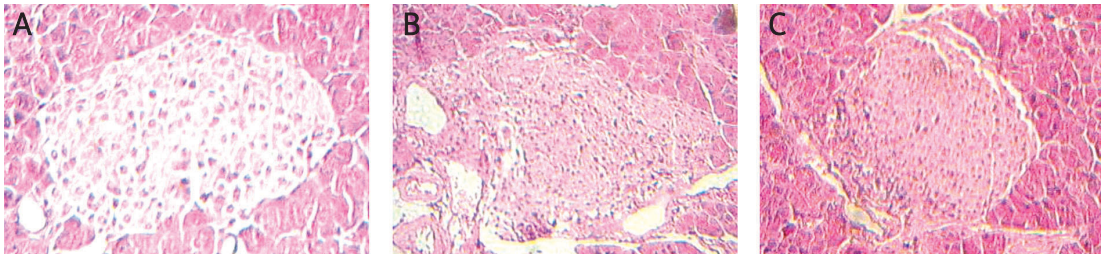

Figure 4. Histopathology study of pancreatic islets. Insulitis was graded 0 to 3, according to infiltration of islets with lymphocytes as none = 0 (**A**), less than 10% = 1 (**B**), 10 to 50% = 2 (**C**), and more than 50% = 3 (was not seen in our study groups). H&E (200 ×)

Discussion

Our data revealed that administration of IMOD™ could ameliorate oxidative stress indicators and proinflammatory cytokines in pancreas of MLD STZ-induced type 1 diabetes in BALB/c mice. However, this effect did not lead to decrease in blood glucose or histological benefit.

MLD STZ-induced diabetes in mice is similar to type 1 diabetes in humans. In this model, infiltration of macrophages and lymphocytes into the pancreas leads to insulitis and activation of mononuclear cells at the site of inflammation then production of nitric oxide (NO), which is cytotoxic to β cells [17]. Insulitis, mononuclear infiltration and inflammatory reactions all happen due to the immunological response that has an essential role in type 1 diabetes. Th1 lymphocytes which belong to CD4+ T cells produce cytokines that primarily stimulate cell-mediated immunity. Both IL-1β and TNF-α as Th1 secreted cytokines have proinflammatory effects. Th2 cells secrete cytokines such as IL-4, IL-6 and IL-1β0, which are believed to play a role in stimulation of humoral immunity [18].

It is now well known that Th1 with its inflammatory cytokines is responsible for β cell damage in type 1 diabetes. In immunological mechanisms of type 1 diabetes, after processing of specific β cell proteins by antigen presenting cells (APCs) such as macrophages and dendritic cells, these proteins act as autoantigens. Presented antigens interact via major histocompatibility complex (MHC) class II molecules on the surface of CD4+ T cells, which leads to production of cytokines such as IL-1β2 from APCs. IL-1β2 promotes CD4+ cells to differentiate into Th1 cells. Th1 characteristic cytokines inhibit Th2 cells' production of their cytokines (IL-4, IL-1β) and activate macrophages and both CD4+ and CD8+ cytotoxic T cells. Finally, these activated cells destroy β cells via their cytokines (TNF-α and IL-1β) or by producing free radicals (H_2O_2, O_2 and NO) especially from macrophages [1].

The idea of immunogenic prevention of type 1 diabetes has been evaluated in many different studies. Th2 cytokines induction [19, 20] as well as Th1 cytokines inhibition [21, 22] has been shown to prevent diabetes in mice. This evidence also suggests that a decrease in Th1/Th2 cell ratio could prevent immunogenic occurrence of diabetes by the aforementioned mechanisms.

On the other hand, previous studies have demonstrated that oxidative stress plays a key role in pathology and progression of type 1 diabetes [23]. Various methods to overcome oxidative stress including antioxidant agents have been recruited to attenuate the development of hyperglycaemia in diabetic mouse models [24, 25].

The present study hypothesis was that IMOD™ as a newly-introduced drug for management of AIDS patients by increasing CD4+ T cells could probably prevent STZ-induced mice from developing type 1 diabetes. IMOD™ contains three herbal extracts, *Rosa canina*, *Tanacetum vulgare* and *Urtica dioica*. *Urtica dioica* leaf extract contains active blends that diminish TNF-α and other inflammatory cytokines [26]. It has been shown that *Urtica* leaf also reduces IL-1β by inhibiting the genetic transcription factor that activates TNF-α and IL-1β in the synovial tissue that lines the joint [27]. The effect of *Urtica dioica* on diabetes has been previously considered [38]. It has been shown that *Urtica* leaf extract could improve type 2 diabetes in STZ-induced mice by increasing secretion of insulin [39]. *Rosa canina* extract possesses abundant antioxidant agents containing vitamin C, vitamin E, flavanoids, and some bioactive compounds which cause this plant to have anti-inflammatory and radical scavenging properties [30]. This herb is used in conventional therapy in different conditions. *Tanacetum vulgare* is also another herb that has antioxidant and anti-inflammatory characteristics and some traditional uses in medicine [31, 32].

However, the present results support the hypothesis indicating positive effects of IMOD™ on oxidative stress and proinflammatory status of the pancreas most probably due to combinational effects of this herbal mixture, but this benefit was not accompanied by complete improvement of blood glucose or reduction of insulitis scores. At the moment, there are no published data on the

mechanisms by which IMOD™ alters the immune system. It has only been shown that IMOD™ increases CD4+ T cells, but the type of CD4+ cells (Th1 or Th2) is not clearly understood.

Regarding the immunological pathogenesis of type 1 diabetes, if Th1 outweigh Th2, the disease will proceed, but if Th2 cells are more differentiated than Th1 ones, the disease will be suppressed. As a limitation of the present study, we did not address which of them was higher in the blood or pancreatic sample of specimens, but our results showed that IL-1β and TNF-α were lower in the pancreas of the IMOD™-treated group. Therefore, it can be concluded that even though IMOD™ reduced Th1 cytokines in the pancreas, total domination of Th1 over Th2 remained stable, resulting in incomplete prevention of diabetes in the IMOD™-receiving group.

Nevertheless, our results are not in congruence with comparable studies [33] reporting that reduction of proinflammatory cytokines and oxidative stress leads to prevention of diabetes and improvement of glycaemia levels. However, these incongruent data might have resulted from combinatory effects of the herbs and the elements present in the mixture of IMOD™. As a matter of fact, as supported by the literature, it is not surprising to observe a reduction of oxidative stress without a hypoglycaemic effect. The same effects in human diabetes have also been reported and are expectable [34-38]. In fact, in the immunogenic model of type 1 diabetes, the blood lowering effect of any compound is mainly mediated through insulin-like effects. Therefore, anti-oxidants or anti-cytokines usually prevent complications or further progress of the disease and thus cannot clear whole disease because some portions of β-cells have been destroyed by STZ irreversibly [3]. Further studies are needed to clarify the effects of IMOD™ on immunological factors to address whether this new agent could be applied in diabetes prevention or therapy.

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