Iranian Journal of Basic Medical Sciences

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IJBMS

Immunotherapeutic effects of pentoxifylline in type 1 diabetic mice and its role in the response of T-helper lymphocytes

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ARTICLEINFO

Article type:
Original article

Article history:
Received: May 5, 2014
Accepted: Feb 4, 2015

Keywords:
Cytokine
Pentoxifylline
Type 1 diabetes mellitus

ABSTRACT

Objective(s): Pentoxifylline is an immunomodulatory and anti-inflammatory agent and is used in vascular disorders. It has been shown that pentoxifylline inhibits proinflammatory cytokines production. The purpose of this study was to investigate the therapeutic effects of pentoxifylline on the treatment of autoimmune diabetes in mice.

Materials and Methods: Diabetes was induced by multiple low dose of streptozotocin (MLDS) injection (40 mg/kg/day for 5 consecutive days) in male C57BL/6 mice. After induction of diabetes, mice were treated with pentoxifylline (100 mg/kg/day IP) for 21 days. Blood glucose levels and plasma levels of insulin were measured. Splenocytes were tested for proliferation by MTT test and cytokine production by ELISA.

Results: Pentoxifylline treatment prevented hyperglycemia and increased plasma insulin levels in the diabetic mice. Aside from reducing lymphocyte proliferation, pentoxifylline significantly inhibited the production of proinflammatory interleukin 17 (IL-17) as well as interferon gamma (IFN-γ), while increased anti-inflammatory cytokine IL-10 as compared with those in MLDS group (diabetic control group).

Conclusion: These findings indicate that pentoxifylline may have therapeutic effect against the autoimmune destruction of the pancreatic beta-cells during the development of MLDS-induced type 1 diabetes in mice.

► *Please cite this paper as:*
Malekifard F, Delirezh N, Hobbenaghi R, Malekinejad H. Immunotherapeutic effects of pentoxifylline in type 1 diabetic mice and its role in the response of T-helper lymphocytes. Iran J Basic Med Sci 2015; 18:247-252.

Introduction

Type 1 diabetes (T1D) results from the destruction of insulin producing pancreatic β cells by a β cell specific autoimmune process (1). Chronic pancreatic inflammation (insulitis) and destruction of islet β-cells in type 1 diabetes is mediated by the immune cells, particularly autoreactive CD4 and CD8 T lymphocytes, B cells, macrophages and dendritic cells (2).

In order to obtain insight into Type 1 diabetes pathogenic mechanisms in humans and to test novel therapeutic approaches for its treatment, different preclinical models of the disease such as spontaneous and accelerated diabetes in the non-obese diabetic (NOD) mice, BioBreeding rats, or diabetes induced in susceptible rodent strains by multiple low doses of streptozotocin (MLDS) are now available (3).

In the pathogenesis of T1D, several proinflammatory cytokines including IFN-ɣ, TNF-α, IL-1, as well as IL-17 (4-6), have been implicated. It is also thought that the production of anti-inflammatory cytokines such as IL-4, IL-10 and TGF-β correlates with protection from T1D (6).

It has been shown that some drugs such as pentoxifylline (PTX) have immunomodulatory and anti-inflammatory activity, which might represent a potential preventive therapy for autoimmune diseases. PTX is a methyl xanthine-derived general phosphodiesterase (PDE) inhibitor that has been available for many years to treat vascular disorder (7).

Phosphodiesterases (PDEs) are a family of enzymes that regulate intracellular levels of the cyclic nucleotides cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) by catalyzing their breakdown to inactive metabolites. Drugs that increase the levels of cAMP, tend to reduce the production of proinflammatory mediators and increase the production of anti-inflammatory mediators by immune cells (8). In recent years, the potential of PTX as an immunomodulatory and anti-inflammatory agent gained interest as it has been shown to effectively suppress the synthesis of TNF-α and other pro inflammatory cytokines such as

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IFN-ɣ and IL-12 *In vitro* and *In vivo* (9-10). PTX has been successfully used for the treatment of experimental autoimmune diseases including experimental autoimmune myocarditis (EAM) (11), experimental autoimmune encephalomyelitis (12), and adjuvant arthritis (13).

In the present study, we hypothesized that Pentoxifylline, due to its anti-inflammatory and immunosuppressive activity, may affect autoimmune diabetes. Consequently, we decided to investigate whether pentoxifylline treatment could prevent the development of MLDS-induced diabetes in mice.

Materials and Methods

Drug and Reagent

Streptozotocin (STZ), citrate buffer, pentoxifylline (PTX), RPMI 1640, L-glutamine, dimethylsulfoxide (DMSO), concanavalin A, MTT ((3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide)) and fetal calf serum fetal were purchased from Sigma-Aldrich (St.Louis, MO). Mouse insulin ELISA kit was obtained from Mercodia (Sylveniusgatan, Sweden). ELISA kits were purchased from BenderMed Systems (Vienna, Austria).

Animals and materials

Male C57BL/6 mice, weighing 20 to 25 g, were housed in a room with a 12-hr light/dark cycle. The mice had free access to tap water and *ad libitum* food.

Animal treatments

Diabetes was induced by multiple low-dose of streptozotocin (MLDS) injection in male C57BL/6 mice. STZ was dissolved in 0.1 M citrate buffer (pH 4.5) and injected intraperitoneally (IP), within 10 min of preparation, at a dose of 40 mg/kg/day for 5 consecutive days. The blood samples were obtained from the tail vein of non-fasted mice, and glucose was measured using a glucometer (Accu-Chech Active). Mice were considered diabetic when their non-fasting blood glucose level was >250 mg/dl (14). Subsequently, the mice were allocated to three therapeutic groups (n=7 per group) (normal control group, MLDS group (diabetic control group) and treatment group). Treatment with PTX (100 mg/kg/day for 21 days, IP) was initiated in treatment group when they were considered diabetic. At the same time, the control groups received saline vehicle alone with the same schedule. Blood was collected by cardiac puncture. Blood volume was about 0.75 ml. Plasma, separated from blood by centrifugation, was stored at -80˚C until insulin assay. Mice were euthanized and their splenic tissues were removed on day 21 for cytokine assay and MTT test.

Quantification of glucose in the blood

Non-fasting blood sugar level was measured in 0, 7, 14 and 21 days after streptozotocin induced diabetes by using a glucometer (Accu-Chech Active).The blood samples were obtained from the tail vein of non-fasted mice (14).

Plasma insulin determination

Before mice were euthanized, blood was collected from each mouse on day 21 in heparinized tubes. Plasma was separated and stored at -80˚C until plasma insulin assay. The insulin level of plasma was determined using a mouse insulin ELISA kit.

Splenocyte culture and cytokine determination

Mice were euthanized and their spleens were removed on day 21 for cytokine production assay. After aseptic removal, spleens were placed in cold Hanks solution and teased apart with a pair of forceps and a needle. A single cell suspension was obtained by passing it through a 200-mesh net and hemolyzed by the buffer solution containing 1 mmol/l Tris–HCl and 1% NH_4Cl (pH 7.2). Subsequently, the macrophage cell content was depleted by incubation of the cell suspension in tissue culture dishes at 37˚ C (air+5% CO_2) to allow these cells to adhere to the bottom of the culture dishes. Remaining free floating cells were seeded on culture dishes at a density of 5×10^6cells/ml in RPMI 1640 with 10% fetal calf serum, and 2 mmol/l L-glutamine. Cell viability was determined by Trypan blue dye exclusion. The splenocytes (5×10^6cells/ml) were treated with 2 µg/ml concanavalin A for 72 hr, and cell supernatants were collected, then the levels of IL-10, IL17 and IFN-ɣ were measured by ELISA kits according to the manufacturers' instructions.

MTT proliferation assay

Mouse splenic lymphocytes (1×10^6cells/ml) were incubated in the absence or presence of concanavalin A (1.25 µg/ml) in a 96-well flat-bottom microculture plat in triplicate for 72 hr. Thirty microliters of 5 mg/ml MTT ((3-(4,5-dimethylthiazolyl)-2,5-diphenyl-tetrazolium bromide)) in PBS was added to each well, and the plate was incubated at 37˚C for 4 hr. The plate was then centrifuged and followed by removal of medium. One hundred microliters of dimethylsulfoxide (DMSO) was then added. After incubation at 37˚ C for 5 min, absorbance was measured spectrophotometrically at 490 nm. The stimulation index (SI) was calculated according to the following formula: SI= OD concanavalin A (Con A) stimulated lymphocyte proliferation/OD spontaneous lymphocyte proliferation without Con A.

Statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test were used for multiple comparisons between groups. Data are expressed as means±SEM. $P<0.05$ was considered significant.

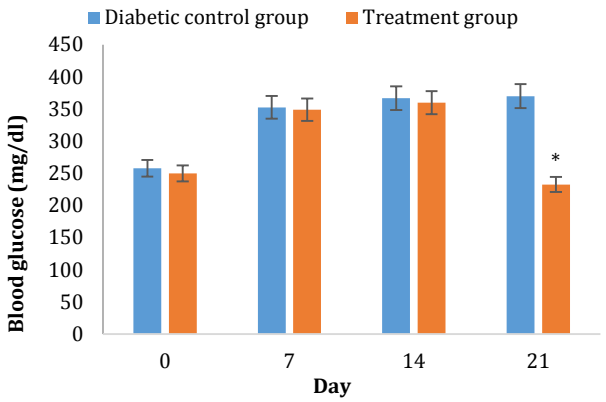

Figure 1. Effect of pentoxifylline treatment on blood glucose levels (* *P*<0.05)

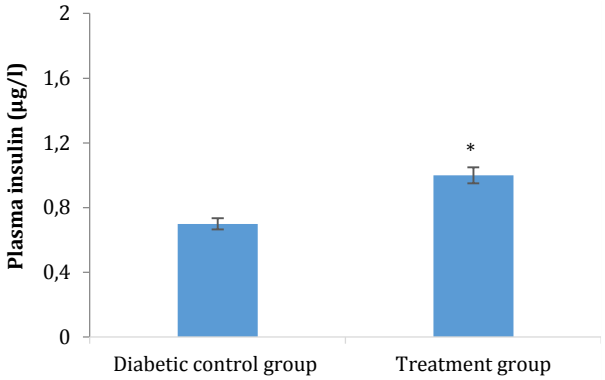

Figure 2. Effect of pentoxifylline treatment on plasma levels of insulin (* *P*<0.05)

Results

Effects of pentoxifylline on MLDS-induced hyperglycemia and plasma insulin level

Administration of PTX for 14 day did not affect blood glucose. All STZ-induced diabetic mice with PTX treatment remained hyperglycemic on day 14, and no significant difference in blood glucose levels was demonstrated between PTX and MLDS(diabetic control group) group. However, the levels of glucose in diabetic mice were significantly reduced after treatment with PTX (*P*<0.05), for 21day, when compared to the values of diabetic control mice (Figure 1).

To evaluate β-cell function in terms of insulin release, we measured the plasma insulin levels on day 21 (Figure 2). PTX prevented the MLDS-induced reduction in plasma insulin, indicating a possible protective effect of PTX against β-cell damage.

Effects of pentoxifylline on cytokine production in type 1 diabetic murine splenocytes

Treatment of mice with PTX significantly decreased MLDS-induced production of IFN- γ and IL-17, while increased IL-10 as compared with those in MLDS group (diabetic control group) (*P*<0.05)(Figure 3).

Effects of pentoxifylline on the proliferation of splenic lymphocytes

The stimulation index (SI) in treatment group with PTX showed a significant decrease as compared with that in MLDS group (diabetic control group) (*P*<0.05)(Figure 4).

Discussion

In rodents, experimental insulin-dependent diabetes can be induced by multiple low doses of streptozotocin (MLDS) (15). This model is a commonly used animal model that has many histological and clinical features similar to those of human type 1 diabetes and involves the participation of macrophages and T cells. Streptozotocin (STZ) is a pancreatic β cell toxin that induces inflammation of the islets by immune cells when it is given in multiple low doses (16).

This model of diabetes offers some advantages, such as simultaneous appearance of diabetes mellitus in all animals and not having immune abnormalities that may complicate studies in spontaneous diabetes in non-obese diabetic (NOD) mice or Bio Breeding (BB) rats. Therefore, this model

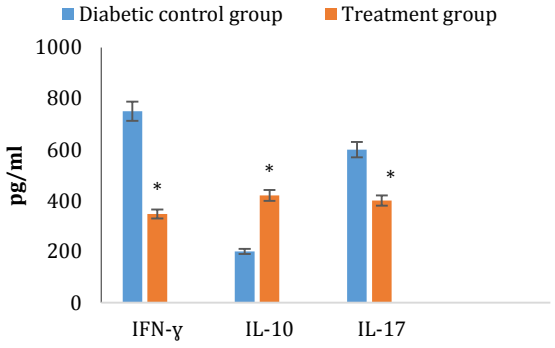

Figure 3. Effect of pentoxifylline on productions of IFN γ ،IL-10 and IL-17 of diabetic murine splenocytes induced by Con A *ex vivo* (* *P*<0.05 versus diabetic control group)

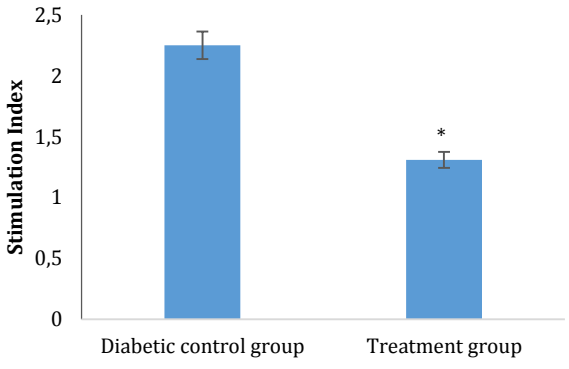

Figure 4. Effects of pentoxifylline on the proliferation of splenic lymphocytes induced by con A after 72 hr (*P<0.05)

as a good model has been applied extensively to investigate the type 1 diabetes mellitus, especially study the immune pathways and modulations of autoimmune insulitis and β cell death (17-18). C57BL/6 mice, also called "C57 black 6" or simply "Black 6" has the advantage of strain stability and easy breeding. The most common application of C57BL/6 mice is to serve as physiological or pathological models for *in vivo* experiments (19). As such, MLDS induced C57BL/6J mice were chosen to evaluate the anti-hyperglycemic activity of PTX and investigate the immunotherapeutic molecular mechanisms of how PTX attenuated the development of T1D.

PTX is commonly used for the treatment of microcirculatory disorders and yields only minimal side effects in patients. In addition, due to its varied immunomodulatory effects, PTX has been used in several autoimmune diseases including rheumatoid arthritis (20), multiple sclerosis (21), and systemic lupus erythematosus (22). Drugs that increase the level of cAMP, including PDE inhibitors, are known to prevent or attenuate inflammatory cell responses (20).

Previous studies have also shown that PTX lowers blood glucose levels in diabetic animals (23), and the results from the present studies also indicated that PTX possessed an anti-hyperglycemic property. More importantly, the present data demonstrated that PTX could increase serum insulin concentrations in type 1 diabetic mice. It has been shown that PTX could reduce (but not abrogate) the insulin requirements or lengthen the “honeymoon” (non-insulin-requiring) period in a study of 21 children with new-onset type 1diabetes (24).

T helper 1 cells produce proinflammatory cytokines (TNF-α, IFN-ɣ, IL-1β, IL-6, and IL-12), which activate macrophages and cytotoxic T cells to destroy β-cells, whereas anti-inflammatory cytokines (IL-4 and IL-10) that are produced by activated T helper 2 cells, prevent β-cell destructive insulitis (25). IL-10 has been shown to prevent the onset of diabetes in mice (26). In response to cytokine stimulation, β-cells generate reactive oxygen species (ROSs) and reactive nitrogen species such as nitric oxide (NO), which facilitate their destruction (27). NO is also synthesized within cytokine-activated macrophages by inducible nitric oxide synthase (iNOS) (28).

More recently, the role of IL-17-producing T cells have been demonstrated in the development of several autoimmune diseases, including multiple sclerosis, rheumatoid arthritis and type 1 diabetes. IL-17 is a proinflammatory cytokine that is detrimental to pancreatic islet cells (29). IL-17 promotes infiltration of neutrophils and macrophages, stimulates the production of other proinflammatory cytokines, including TNF-α, IL-1β, IL-6 and IL-12, by activated macrophages and induces nitric oxide release from β cells that interferes with their function and cause β-cell destruction (30, 5).

It has been assumed that PTX similar to cAMP elevating agents, selectively suppresses the production of T helper type 1(IL-2, IFN-ɣ), but not T helper type 2(IL-4, IL-6, IL-10) cytokines (31). However, it has also been demonstrated that the effect of PTX on cytokine production is cell-specific (32).

The present study shows that PTX is not only able to inhibit the release of proinflammatory cytokines (IFN-ɣ and IL-17), but can also increase the production of anti-inflammatory cytokines (IL-10).

Since PTX has been found to be able to inhibit the release of proinflammatory cytokines (33), and favor Th2 response (34), it may indirectly interfere with the induction of iNOS. It has been shown that PDE inhibitors elevate cAMP, prevent or attenuate inflammatory cell responses (35) and suppress proliferation of lymphocytes (36). Our findings are consistent with previous studies, showing that PTX treatment prevented lymphocyte proliferation.

Conclusion

In summary, results of the present work demonstrated that the suppressive effect of the PTX treatment on T1D was accompanied by decreased blood glucose level, increased plasma insulin level, suppression of T cell proliferation, down-regulation of Th1 and Th17 cytokines (IFN-ɣ and IL-17) and increase in the production of IL-10 in supernatant of splenic culture of the treated mice. It seems that pentoxifylline treatment has a therapeutic effect against MLDS-induced diabetes in mice.

Acknowledgment

We would like to sincerely thank the members of the Faculty of Veterinary Medicine and Urmia University Research Council for the approval and support of this research. The results described in this paper were part of student thesis. This project is financially supported by Educational Vice President of Urmia University, Urmia, Iran.

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