Herbal flavonoids inhibit the development of autoimmune diabetes in NOD mice: proposed mechanisms of action in the example of PADMA 28

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Abstract

Padma® 28 (PADMA Inc. Schwerzenbach, Switzerland) is a herbal multi-component preparation, which is produced as a commercial product according to international quality guidelines in Switzerland. Each batch of components is validated regarding identity and specifications, which are deposited at the Swiss authorities for the authorization and determination of insulin levels. The herbal formulation is rich in secondary plant substances, such as essential oils, flavonoids, and tannins.

Padma 28 has been successfully used in different chronic inflammatory diseases and has also shown anti-inflammatory effects in various in vitro studies. Inflammation plays an important role in the pathogenesis of type 1 diabetes mellitus, an autoimmune disorder characterized by the destruction of pancreatic beta-cells, resulting in insulin deficiency and hyperglycemia. Female non-obese diabetic (NOD) mice spontaneously develop autoimmune diabetes and are considered an experimental model for human type 1 diabetes mellitus. Histological examination of the pancreas from NOD mice revealed characteristic mononuclear cell infiltrations of the islets, predominantly by T cells, resulting in autoimmune insulinitis and low functional islets. As in many other autoimmune diseases, cytokines secreted by macrophages and lymphocytes are also thought to be involved in the development of diabetes. While the administration of INF-γ can cause or accelerate autoimmune disease in NOD animals, anti-inflammatory cytokines such as interleukin IL-10 may have protective effects. In mice, IL-10 produced by the Th2 subset of CD4+ T-helper lymphocytes, IL-10B lymphocytes, and macrophages inhibited proliferation of Th1 cell-derived INF-γ and IL-2 by blocking co-stimulatory functions of accessory cells. Several agents capable of suppressing the development of diabetes in laboratory animals, possibly due to a modulation of the cytokine production (imbalance between Th1/Th2 responses), have been described. Since reactive oxygen and nitrogen species are also implicated in the pathophysiology of diabetes and since oxidants were found to synergize with proteases, membrane-damaging agents and with certain cytokines to injure mammalian cells, it was postulated that herbal preparations rich in anti-oxidants, bioflavonoids and anthocyanidins, might be able to inhibit the development of diabetes.

To investigate whether secondary plant substances such as herbal flavonoids are able to prevent the development of autoimmune diabetes, we analyzed the formula Padma 28 in a pre-clinical model of the autoimmune disorder type 1 diabetes mellitus.

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Materials and Methods

Padma 28 extracts

Padma 28 is produced by PADMA Inc. according to GMP guidelines in Switzerland. It is distributed in several European countries as well as in Canada and the USA for oral use. The formula contains: Bengal quince (Aegle marmelos (L) Correa) 20 mg, allspice (Pimenta dioica (L) Merr) 25 mg, colombine (Aquilegia vulgaris L) 15 mg, calcium sulphate 20 mg, calendula flower head (Calendula officinalis L) 5 mg, natural camphor (Cinnamomum camphora (L) Sieb.) 4 mg, cardamom fruit (Elettaria cardamomum (L) Maton) 30 mg, clove (Syzygium aromaticum (L) Merrit et L.M. Perry) 12 mg, costus root (Saussurea lappa C.B. Clarke) 40 mg, kempferia galanga rhizome (Kaempferia galanga L) 10 mg, lettuce leaf (Lactuca sativa L) 6 mg, Iceland moss (Cetraria islandica (L) Ach.) 40 mg, liquorice root (Glycyrrhiza glabra L) 15 mg, neem fruit
NOD mice

Nine to fifteen 5-6 week old female NOD mice were tested per group. The animals were purchased from Harlen Breeding Facility, Jerusalem, Israel. The animals were fed standard laboratory chow ad libitum, and were kept in an SPF animal house facility.

Padma 28 administration and development of overt diabetes

Five groups of NOD mice were used in a trial that lasted 24 weeks. Group I (15 animals) was left untreated, Group II (15 animals) served as control and was administered 0.2 mL saline i.p. 5 times during the first week, and then received one injection of saline per week for a total of four weeks. One month later, 0.3 mL of saline was given once a week and two weeks later, 0.25 mL of saline was given once a week for three additional weeks. Group III (15 animals) was administered i.p. 0.3 mL (3 mg dry weight of Padma 28) 3 times during the first week. Additional injections of Padma 28 were given according to the schedule described for group II. Group IV (10 animals) was administered 250 mg/kg Padma 28 s.c. according to the schedule described for group II. Group V (9 animals) received 7 mg Padma 28 p.o. via a gastric tube 5 times during the first week and then according to the time schedule described for group II.

All mice were monitored daily, and urine was analyzed for the presence of glucose once or twice a week using a standard glucose stick (Medi-Test Combi 9 Macherey-Nagel, Duren, Germany). The development of overt diabetes was confirmed and considered positive only if on two separate occasions the level of glucose in the urine was greater than 1,000 mg/dL.

Intraperitoneal glucose tolerance test

Mice that had survived for 24 weeks without developing overt diabetes (glucosuria negative) were administered D-glucose at 1g/kg body weight i.p. Blood glucose levels were determined with a Glucometer Elite apparatus (Bayer Diagnostics, Elkart, IN, USA) at 0 and 60 min after glucose injection. Animals with glucose levels greater than 140 mg/dL at 60 min were considered as having latent diabetes.

Quantitative analysis of insulin in pancreas

Animals were sacrificed at week 24, the pancreas was immediately removed and the head portion was frozen at -70°C for the subsequent analysis of insulin content following acid-extraction extraction, as described previously.  

The remaining part of the pancreas was fixed in 10% phosphate-buffered formalin and embedded in paraffin. Multiple consecutive sections of 5 μm were stained with hematoxylin and eosin for histology or prepared for immunohistochemical analysis, as outlined below.

Insulin content was determined by radioimmunoassay, using anti-insulin-coated test tubes (ICN Pharmaceuticals, Costa Mesa, CA, USA) and 125I-insulin (Linco Research, St. Charles, MO, USA). The routine intra-assay coefficient of variation (CV) was 4-6%, the inter-assay CV was 6-10%. Rat insulin standards (Novo-Nordisk, Bagsvaerd, Denmark), were used for the determination of mouse insulin-like immunoactivity.

Immunohistochemical analysis for the presence of insulin in the pancreas

The presence of diabetes was also confirmed by histological examination of the pancreas, as described previously. Briefly, de-paraffinized sections were rinsed with phosphate buffered saline (PBS) and endogenous peroxidase was blocked by exposure to 3% H2O2 for 15 min at room temperature. Insulin-containing cell sections were then incubated with a 1:100 dilution of guinea-pig anti-insulin antibody (Sigma, Rehovot, Israel) at 37°C for 1 h, and detected using a streptavidin-biotin peroxidase complex developed with aminoethylcarbazole substrate (Zymed, San Francisco, CA, USA). Two sections per animal were evaluated by light microscopy at x200 magnification.

Evaluation of cytokines production

Cytokine production was evaluated in plasma and in supernatant fluids from cultured spleen cells. Spleen cells were stimulated with 2.5 μg/mL concanavalin A (Con A) and cytokines were analyzed after 24 and after 48 h.

The levels of the Th1 cytokines IL-12 and interferon (INF)-γ and of the Th2 cytokines IL-10 and IL-6 were measured by a solid phase ELISA (R & D, Minneapolis MN, USA) using a quantitative sandwich enzyme immunoassay technique as described previously.  

Proliferative response detected by 3H-thymidine incorporation

Spleen cells obtained from NOD mice were teased into single cell suspensions on a nylon mesh and then suspended in Hank's buffer. Cell viability was determined by trypan blue exclusion. A spleen cell suspension from each mouse was prepared in RPMI-1640 (Biological Industries, Bet Haemek, Israel) containing 10% fetal calf plasma (FCS) (GIBCO, Grand Island, NY, USA). Cells were washed twice, counted and were seeded in the concentration of 5x10^5 cells per well in triplicate. They were cultured for three days in flat bottomed 96-well micro plates in the presence of 2.5 μg/mL Con A (Bio Makor, Rehovot, Israel). Twenty hours before harvesting, 1 μCi 3H-thymidine (specific activity 5 mCi/mMol, Nuclear Research Center, Negev, Israel) was added. The proliferative response was determined by measuring the incorporation of 3H-thymidine. The data were expressed as an index of Con A treated/unstimulated cells.

Fluorescence-activated cell sorter analysis

Single cell suspensions of individual spleen cells from NOD mice were analyzed for cell surface markers as follows: 50 μL of cell suspension (10^6 cells) in PBS containing 0.1% sodium azide and 2% FCS were mixed with 10 μL fluorescent isothiocyanate (FITC)-conjugated monoclonal anti-CD4 antibodies (recognizes T-helper cells) and anti-CD8 antibodies (recognizes cytotoxic T cells). Anti-mouse CD25 (expressed by activated T and B lymphocytes and NK cells but not by resting T, B, or NK cells), anti-mouse CD69-FITC (recognizes activated T, B and NK cells) and anti-Mac-1 (recognizes a differentiation antigen stimulated on mononuclear phagocytes and granulocytes) were purchased from Pharmigen, San Diego, CA, USA. Hamster anti-mouse CD69 was purchased from Serotec Oxford, UK. The cell mixtures were incubated for 30 min on ice, washed twice, and resuspended in 0.2 mL PBS containing 0.1% sodium azide. After correcting for non-specific staining, the percentage of cells staining positively for the various markers was determined by FACS analysis (FACS, 440, Becton-Dickinson Franklin Lakes, NJ, USA).

Statistical analysis

The Mann-Whitney U test was used for between group comparisons. P<0.05 was considered significant.
Results

Effect of Padma 28 on the survival and development of overt diabetes in NOD mice

After 24 weeks, 100% of the animals injected i.p. with Padma 28, 80% of the animals injected s.c. with Padma 28, and 33% of the animals administered Padma 28 p.o. were free of diabetes (Figure 1). On the other hand, only 20% of the animals in the untreated and in the saline control groups were diabetes-free. In Padma 28 treated animals that developed autoimmune diabetes, the onset of the signs of the disease was delayed by four days in the p.o. treated animals (first signs on day 16 compared to day 12 in controls) and by one week in the s.c. treated animals (first signs appeared on day 18).

Glucose tolerance test

All the animals that had survived by the end of the trial and showed no glucosuria underwent an i.p. glucose tolerance test. Overt diabetes was confirmed only if on two occasions the glucose levels in urine exceeded 1,000 mg/dL. Of the 4 untreated animals which had survived, 2 were diabetes free and 2 animals had latent diabetes. In the saline treated group, 2 of 4 mice had overt diabetes, and one animal had latent diabetes. In the Padma 28 group treated i.p., 4 of the 11 surviving animals had latent diabetes and 7 were diabetes free. In the group receiving Padma 28 s.c., 8 survived, none had overt diabetes and 3 had latent diabetes. Finally, in the group administered Padma 28 p.o., only 3 survived for 24 weeks and all had latent diabetes.

Quantitative analysis of insulin in the pancreas

Whereas a very marked depletion of insulin content was observed in untreated animals with an average of 0.198 pmol/mg tissue (n=4), the pancreata of normal mice (n=4) and of mice treated i.p. with Padma 28 (n=5) had a large insulin content of 300 pmol/mg.

Staining of pancreatic islets with anti-insulin antibodies

While only a very few insulin-positive beta cells were found in untreated mice, the surviving animals administered Padma 28 exhibited preserved islet morphology with multiple insulin-containing cells. Thus, the histochemical analysis paralleled the quantitative analysis of insulin in the pancreas (data not shown).

The proliferative responses of spleen cells to Con A

As T cells are involved in the pathophysiology of type 1 diabetes, the proliferative response of T cells derived from control and from Padma 28 treated animals to the non-specific mitogen Con A was determined. Figure 2 shows an apparent reduction in cell proliferation from animals treated i.p., s.c. and p.o. with Padma 28. Although these values did not reach statistical significance due to a high variance in the control group, nevertheless an anti-proliferative effect of the herbal mixture may be suspected, which is also supported by the results obtained in previous studies.

Cytokines in spleen cell supernatants and in plasma

Upon termination of the trial, the levels of several key cytokines were determined in the plasma and in supernatant fluids from cultured spleen cells. Spleen cells were cultured and stimulated with Con A, and cytokines were measured after 24 and after 48 h. A significant elevation of IL-10, IL-6, IL-4 and INF-γ (Figure 3a-d) was observed. Among these were the Th2 cytokines IL-10, IL-6, and IL-4, which are known to have anti-diabetic effects in autoimmune diabetes. Results after 24 h were similar but less pronounced.
observed in the supernatant fluids from unstimulated and Con A-stimulated spleen cells obtained from Padma 28 treated mice. In plasma, IL-10 and IL-6 were increased in treated animals as a trend, although only an increase of INF-γ in the s.c. group and a decrease of IL-12 in the i.p. group reached statistical significance (Table 1).

Phenotypes of spleen cells involved in autoimmune diabetes in NOD mice

Since autoimmune insulitis is thought to be caused by T cells, the phenotypes of lymphocytes involved in the development of diabetes were analyzed. Spleen cells from Padma 28 treated and untreated NOD mice were analyzed by direct immunofluorescence staining for surface markers. The following phenotype markers were tested: CD4, CD4CD25, CD25, CD8, CD69, CD8CD69, and CD11b. A significant decrease in CD8 cells was found in animals treated i.p. with Padma 28 compared to controls (Figure 4). CD8 cytotoxic cells represent T cells involved in the destruction of the islets. On the other hand, Padma 28 had no effect on the percentages of CD4.

Discussion

Padma 28 is a complex mixture of secondary plant substances. Essential oils make about 1.1% (v/w) and include, among others, eugenol, camphor, 1,8-cineole, geraniol and thymol, which are known to have antiphlogistic and locally analgesic effects, and to inhibit platelet aggregation. Flavonoids are present in the polyherbal preparation at about 0.16% (w/w) and include among others isoflavonol, quercetin, luteolin, and apigenin, for some of which anti-hypertensive and anti-inflammatory effects, the ability to improve endothelial function, and oxygen radical scavenging and cytotoxic properties have been shown. Gallotannins and catechin tannins, present in the formula at about 1.1% (w/w), are known to have locally anti-inflammatory and anti-oxidative effects.

Different parameters of diabetes mellitus, such as glucosuria, impaired glucose tolerance, insulin content of the pancreas, islet morphology and survival were all favorably influenced by i.p. or s.c. administration of aqueous extracts from Padma 28.

Although the oral route of administration has shown good efficacy in peripheral arterial occlusive disease (PAOD), as well as in various clinical studies and recently also in a meta-analysis, in this NOD model the onset of clinical studies and recently also in a meta-occlusive disease (PAOD), as shown in various quantities in Padma 28, either unaltered or following partial biodegradation, seem to be able to change the cytokine profiles. In our study, as well as in previous works, the herbal preparation induced the generation of the anti-inflammatory cytokine IL-10. The protective effect of IL-10 against autoimmune insulitis is supported by various authors. Additional cytokines, and especially the balance between Th1 and Th2 agents, play a role in autoimmune diabetes, whereby a dominant Th2 immune reaction exerts a protective effect. Animals treated i.p. or s.c. with Padma 28 extract also showed elevated levels of other Th2 cytokines such as IL-4 and IL-6 (Figure 3a-c). However, the production of the proinflammatory marker INF-γ also increased, whereas a change was found in the levels of other pro-inflammatory cytokines. These results were also observed in the plasma of s.c. treated animals (Table 1). In the plasma of Padma 28 treated mice a decrease in Th1 cytokine IL-12 was found, which reached statistical significance in the i.p. group. The changes in cytokine levels indicate a shift from Th1 to Th2 type immune reaction. This may be due to the sequestration of slowly biodegradable flavonoids and anthocyanidins contained in Padma 28 within macrophages in the reticuloendothelial system, leading to the generation of the Th2 cytokines IL-10 and IL-6, but also to a sharp decrease in IL-12, known to depress the levels of Th2 cytokines.

While non-destructive lesions were found to be associated with the Th2 cytokines IL-4, IL-10 and TGFβ, several investigations have shown that autoimmune diabetes can be regulated by a delicate balance between the pro-inflammatory Th1 cytokines INF-γ, TNFα, IL-2, IL-12, low frequencies of IL-4, and an increase in the expression of IL-1, TNFα and INF-γ. It was also shown that treatment with IL-4 or

Table 1. Changes of cytokines in the plasma of Padma 28 treated mice (means ± SEM, significant values marked in bold and underlined, P<0.05).

<table>
<thead>
<tr>
<th>Group</th>
<th>IL-12 (pg/mL)</th>
<th>INF-γ</th>
<th>IL-10 (pg/mL)</th>
<th>IL-6 (pg/mL)</th>
<th>IL-4</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>48.3±20</td>
<td>&lt;4.1</td>
<td>&lt;6.8</td>
<td>10.26±2.1</td>
<td>&lt;3.4</td>
</tr>
<tr>
<td>Padma 28 i.p.</td>
<td>7.1±1.3</td>
<td>4.2±0.07</td>
<td>19.4±13.3</td>
<td>8.89±4.21</td>
<td>&lt;3.4</td>
</tr>
<tr>
<td>Padma 28 s.c.</td>
<td>18.5±4.4</td>
<td>10.3±1.7</td>
<td>12.45±5.65</td>
<td>16.63±3.02</td>
<td>&lt;3.4</td>
</tr>
<tr>
<td>Padma 28 p.o.</td>
<td>21.5±1.5</td>
<td>&lt;4.1</td>
<td>&lt;6.8</td>
<td>23.45±9.6</td>
<td>&lt;3.4</td>
</tr>
</tbody>
</table>

Figure 4. CD8 cells in spleens of Padma 28 and control mice. Reduction was significant in i.p. treated animals.

Figure 5. Possible mechanisms by which plant preparations such as Padma 28 might exert anti-diabetic effects.
IL-10 protected NOD mice from diabetes\textsuperscript{4,5,9} and thus the elevation of these two cytokines in Padma 28 treated mice may be one mechanism of protection against autoimmune diabetes. Noting this, in contrast to most reports, Serreze et al. discuss the possibility that a Th1-Th2 shift and an increase in IL-10 levels may be the outcome rather than the cause of protection of NOD mice from diabetes.\textsuperscript{10}

In addition to the role of cytokines and Th1/Th2 balance in autoimmune diabetes, the involvement of cellular immune systems in the pathogenesis of pancreatic islet destruction is well established.\textsuperscript{11} The role of macrophages in the pathophysiology of diabetes in NOD mice is supported by studies showing that diabetes can be inhibited by macrophage transfer.\textsuperscript{12} An analysis of the T-cell subsets did not show significant changes in CD11b, CD4, CD25, CD25CD4 and CD69 cells while CD8 cells were reduced following the i.p. treatment (Figure 4). CD8 cells are involved in the development of diabetes as well as in beta cell destruction.\textsuperscript{13} Such a reduction in CD8 cells might be induced by antibodies directed against CD8, resulting in protection from diabetes as shown by Solomon and Sarvetnick.\textsuperscript{14}

We demonstrated a protective effect induced by the i.p. and s.c. routes of administration of Padma 28 against the development of diabetes in NOD mice. The study also shows different mechanisms by which flavonoids might act on cellular cytokine levels that are at least partly responsible for the overall effect of Padma 28.

The results thus support a pleiotropic mode of action for the herbal multi-compound also in autoimmune diabetes, as was also implied by various \textit{in vitro} and \textit{ex vivo} studies employing the whole formula containing other secondary plant substances such as essential oils, flavonoids, and tannins. We propose a pleiotropic mode of action for complex herbal substances inhibiting the development of autoimmune diabetes as summarized in Figure 5.

**Conclusions**

The results of this study indicate that an aqueous extract of Padma 28 has the ability to suppress the development of autoimmune diabetes in female NOD mice. The levels of IL-10 and IL-6 were increased in i.p. and s.c. treated mice and with i.p. administration the proportion of CD8 cells, known to be effectors in beta cell destruction, was reduced. The data suggest a shift of the Th1/Th2 balance towards Th2 immune response which seems to be protective in the development of diabetes. These results support a multi-target mode of action for the complex herbal preparation.

Further studies are needed to elucidate in more detail the protective effects of herbal flavonoids and other secondary plant substances in autoimmune diabetes.

**References**


27. Bischoff SC. Quercetin: potentials in the


