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Curcumin Ameliorate Diabetes Type 1 Complications through Decreasing Pro-inflammatory Cytokines in C57BL/6 Mice

Yaser Jafari Khataylou¹, Somayeh Ahmadiashar², Reza Rezaei¹, Saeid Parsamanesh¹, and Golbahar Hosseini¹

¹ Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

² Department of Microbiology, Faculty of Veterinary Medicine, University of Urmia, Urmia, Iran

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ABSTRACT

Type 1 diabetes is a chronic autoimmune disease of beta cells in the islets of Langerhans, which are responsible for making insulin. Even with insulin therapy, inflammatory complications will develop in the long term.

The present study examines changes in serum levels of interleukin (IL)-6, IL-17, IL-10, tumor necrosis factor (TNF)- α , interferon (IFN)- γ , C-peptide, Insulin as well as fasting blood sugar (FBS) in control, diabetic and diabetic treated with curcumin groups. Thirty inbred C57BL /6 mice were randomly divided into three groups of 10 mice: group A consisted of healthy mice receiving citrate buffer, group B included a group of diabetic mice, and group C was a group of diabetic mice treated with curcumin. The cytokine levels were measured in the supernatant of stimulated splenocytes using enzyme-linked immunosorbent assay (ELISA). Radioimmunoassay was used to measure insulin and c-peptide levels. The FBS was measured by an automatic glucometer device.

The levels of IL-6, IL-17, and IFN- γ , as well as FBS, was significantly decreased in the treated group with curcumin compared to the diabetic group mice ($p < 0.05$). TNF- α levels were also low, but the difference was not significant. IL-10, plasma C-peptide, and insulin significantly increased in the supernatant of stimulated splenocytes of treated diabetic group than in the diabetic group ($p < 0.05$).

According to the results, this study supports the anti-diabetic and anti-inflammatory effects of curcumin; however, more studies are needed to investigate the effects of curcumin and the dose-response relationship in this disease.

Keywords: Anti-inflammatory agents; C-peptide; Curcumin; Inflammation; Mice; Type 1 diabetes mellitus

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease in which β cells are destroyed in the islets of

Langerhans that are responsible for making insulin.¹ As β cells were destroyed in the islets of Langerhans, patients with T1D lost control of blood glucose, which can lead to severe conditions such as ketoacidosis

Corresponding Author: Yaser Jafari Khataylou, PhD;
Department of Pathobiology, Faculty of Veterinary Medicine,

University of Tabriz, Tabriz, Iran. Tel: (+98 914) 3457 781, Fax:
(+98 41) 3637 8743, E-mail: y.jafari@tabrizu.ac.ir

severe hyperglycemia,² and secondary complications even with treatment replacing insulin.^{3,4} The disease, like other organ-specific autoimmune diseases, is caused by impairment in the regulation of immune responses.⁵ It has been widespread in studies in which T1D occurs frequently in children and adolescents, but the age effect has been shown less in recent studies.⁶

C-peptide is released at the equimolar concentration with insulin from the pancreas; however, its half-life is longer than that of insulin, and its systemic concentration in blood circulation is approximately five times higher than that of insulin.⁷ This peptide is also an excellent indicator to investigate the expansion of β cells destruction. C-peptide is suitable for subtraction between T1D and type 2 diabetes (T2D), particularly in the long term, since there is no C-peptide in T1D.⁸

Immunemediators in T1D play a broader role than it was previously considered to induce and enhance the immune response against pancreatic β cells. The progression of diabetes mellitus occurs when one or more regulatory mechanisms of the immune system are disrupted, resulting in the reaction of autoreactive T cells in the pancreatic islets β of Langerhans, and formation of an immune-inflammatory cascade in these islets (insulinitis) as well as maximization of the destruction of these cells.⁵

Among T cells, T helper 1 (TH1) plays a role in cellular immunity and production of inflammatory cytokines such as Interleukin-2 (IL-2), interferon-gamma (IFN- γ), and tumor necrosis factor (TNF- α). T helper 2 (TH2) cells play a role in humoral immunity, contribute to lymphocytes B, and release cytokines such as IL-4 and IL-10. The balance between the cytokines produced by TH1 and TH2 cells secreted by immune cells in the islets of Langerhans is important in diabetes. The collapse of the balance and the tendency toward cytokines such as IFN- γ and TNF- α cause the development of inflammatory insulinitis and diabetes.⁹ IL-1, TNF- α , TNF- β , and IFN- γ (in pico and nano concentrations) have cytotoxic effects on Langerhans β cells. It should be noted that these cytokines can prevent the production and secretion of insulin, but this effect can considerably be eliminated after removing cytokines. Curcumin, a natural polyphenol, is found in the *Curcuma longa* rhizome and has anti-inflammatory, antioxidant, antineoplastic and chemopreventive activities.¹⁰⁻¹⁴ The effect of the anti-inflammatory mediators of curcumin through the reduction of nuclear factor transcription κ B (NF- κ B), IL-6, TNF, inducible

nitric oxide synthase (iNOS), adhesion molecules, IL-8, cyclooxygenase-2 (COX-2), matrix metalloproteinase-9 (MMP-9), and 5-lipoxygenase (5-LOX) is confirmed. It has been seen that curcumin co-crystallizes after attachment to the active site of 5-lipoxygenase.¹⁵

In this study, owing to inflammatory complications caused by autoimmune T1D as a result of inflammatory cytokines, and the anti-inflammatory effect of curcumin, the role of curcumin in reducing the inflammatory complications of this autoimmune disease and its role in lowering the fasting blood sugar (FBS) and increasing C-peptide and plasma insulin are shown.

MATERIALS AND METHODS

Animal

This study was conducted on C57BL/6 inbred mice aged 6 to 8 weeks that were purchased from the Pasteur Institute of Iran and randomly divided into 3 groups of 10 mice. Group A consisted of healthy mice that received citrate buffer. Group B included a group of diabetic mice, and Group C was a group of diabetic mice treated with curcumin. The experimental method was carried out by the University of Tabriz Animal Ethical Committee (Ref No. IR.TABRIZU.REC.1398.002).

Diabetes Induction in C57BL/6 Mice and Treatment Protocol

Induction of diabetes was carried out through streptozotocin (STZ, Sigma, Germany) at 50 mg/kg for 5 consecutive days intraperitoneally (IP), and fasting for 4 hours before administration of each STZ dose of the mice. Ten minutes before STZ injection, 50 mg/kg of it was dissolved in 200 μ L citrate buffer with PH=4.5, and then it was used. When FBS reached 300 mg/dL, the mice were considered diabetic.¹⁶ One day after the last induction dose of STZ (the sixth day), curcumin (Sigma, Germany) was injected IP at a dose of 30 mg/kg for 14 days, blood samples were collected, and their serum and plasma were separated to measure IL-6, IL-17, IL-10, TNF- α , IFN- γ , FBS (serum), C-peptide, and insulin (plasma). One day after the last dose of curcumin, blood sampling was done through the tail vein of mice (it was done for all 3 groups of mice) to separate serum and plasma. Plasma was used to measure c-peptide and insulin with the radio-immune assay kit and serum to measure IL-6, IL-17, IL-

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10, TNF- α , and INF- γ by the ELISA commercial kit (the sensitivity was 5.0 pg/mL for IL-10 and IL-6, 1.6 pg/mL for IL-17 and 5.3 pg/mL for INF- γ and TNF- α).¹⁶

Evaluation of Diabetes in Mice

One day after the last induction dose of curcumin (the twentieth day), the FBS level in venous tail blood was measured using a Glucometer (ACCU-CHEK, compact plus, Ireland), one day after the last injected dose of curcumin. To measure plasma insulin and C-peptide, a commercial kit (Rat Insulin ELISA kit, CAT # 1NSKR020, Crystal chem. Inc, Chicago, IL) and a radio-immuneassay commercial kit (cat # RCP-21K, Linco Res. Inc., st.charles, MO) were used according to the manufacturer's protocol (the sensitivity was 1.0 ng/mL for insulin and 0.5 ng/mL for C-peptide). To measure IL-6, IL-17, IL-10, TNF- α , and INF- γ , the ELISA commercial kit (BENDERMED Co.-Austria) was used. The mice were kept fasting for 4h before evaluation. The sensitivity was 5.0 pg/mL for IL-10 and IL-6, 1.6 pg/mL for IL-17, and 5.3 pg/mL for INF- γ .

Pancreas Extract Preparation

One day after the last induction dose of curcumin (the twentieth day), six male C57BL/6 mice (6-8 week-old) were sacrificed by cervical dislocation, and the pancreases were removed and homogenized in chilled phosphate buffer saline (PBS) containing protease inhibitors (Sigma, Germany). The homogenates were centrifuged in a two-step process at 3000 rpm for 10 min at 4°C followed by 20 min at 12000 rpm. The supernatant was collected and evaluated for protein concentrations using the Bradford protocol. The extract was stored at -80°C as aliquots until it was used.¹⁷

Stimulation of Spleen Cells for Evaluation of the Cytokines

One day after the last induction dose of curcumin (the twentieth day), all 3 groups of mice were sacrificed by cervical dislocation, and their spleens were picked and placed in Petri dishes containing 5 mL complete RPMI 1640 medium (Roswell Park Memorial Institute) (Sigma Co., USA), which was supplemented with 10% fetal bovine serum (Gibco Co., Germany). The samples were minced into several pieces, then filtered through a cell strainer (0.1 mm) to eliminate clumps and debris. Cell suspensions (3 mL) were then placed in a

centrifuge tube, and a 3-mL high-density Ficoll (Sigma, Germany) was put under it by letting the Ficoll flow under the cell suspension, followed by centrifugation at 600g for 15min at 4°C. The cell pellets were then removed and washed with PBS twice. The cells were plated in 24-well plates (2×10⁶ cells per well) in RPMI 1640 supplied by 10% fetal bovine serum with or without pancreas extract (50 μ g/mL) as a stimulator (We used two controls, positive one with phytohemagglutinin (PHA) stimulation and the negative one without stimulation) for 72h in 5% CO₂.¹⁸

Measurement of Cytokine Levels from Splenocytes

Commercially ELISA kits (Bender Med Co., Austria) were used to measure concentrations of each cytokine according to the kit manufacturer's instructions, the concentrations of the cytokines were expressed as average (pg/mL), and the sensitivity was 5.0 pg/mL for IL-10 and IL-6, 1.6 pg/ml for IL-17 and 5.3 pg/mL for INF- γ . The average OD (optical density) was calculated, and the standard curve was plotted on logarithmic paper. Using a standard curve, the amount of each of the cytokines in the sample was determined as pg/ml, and the standard curve was depicted between 15.6-1000 pg/mL (IL-10, INF- γ) and 7.8-500 pg/ml (IL-17, IL-6, and TNF- α) of cytokines in the solution. The criterion for ensuring work accuracy is a lack of difference of more than 20% of each sample with the average calculated. It should be noted that since the test sample is diluted in a 1 to 2 ratio in the third step, the final concentration of cytokines is determined by doubling the numbers obtained from the standard curve. The results from five subjects were expressed as Mean \pm SEM.

Statistical Analysis

Statistical analyses were conducted; using the SPSS software 19.0 and one-way ANOVA. Additionally, the mean comparison was performed using Tukey HSD for parametric tests and non-parametric tests (repeated measure) for F.B.S data. Significance was considered at $p < 0.05$, and the data were reported as Mean \pm SEM.

RESULTS

Measurement of Cytokine Levels from Splenocytes

T1D is a chronic inflammatory disease caused by the destruction of β cells through immune system function. Many studies have noted the crucial role of

NF- κ B in the destruction and function of β cells in T1D. Furthermore, it has been demonstrated that the activation of NF- κ B is induced by some cytokines such as IFN- γ , IL17, TNF- α , and IL-6, and pro-inflammatory cytokines play a key role and most likely trigger and release inflammatory cascades damaging pancreatic cells. Moreover, changes in pro-inflammatory cytokine levels (especially IL-17, TNF- α , IFN- γ , and IL-6) in patients with T1D indicate its importance as a potential pathogenesis indicator and the vital role of these cytokines in promoting complications and problems caused by autoimmune diabetes.^{9,10} In this regard, our results showed that the levels of IL-6, IL-17, IFN- γ and TNF- α in the supernatant of stimulated splenocytes of diabetic mice (Group B) was markedly higher than that of the healthy control group (Group A), which was significant ($p < 0.05$). However, the IL-10 level in the supernatant of stimulated splenocytes of diabetic mice was significantly lower than that of the healthy control group ($p < 0.05$), and IL-6, IL-17, and IFN- γ were significantly lower in diabetic mice treated with curcumin (Group C) than in untreated diabetics (Group B) ($p < 0.05$).

Additionally, TNF- α levels were low, but this difference was not significant ($p > 0.05$). The level of IL-10 in diabetic mice treated with curcumin was significantly higher than that of the untreated diabetic group ($p < 0.05$), which was significant (Figure 1).

Measurement of FBS

The destruction of β cells leads to insulin failure and patients develop dangerous hyperglycemia, which is the most important factor in type 1 diabetes, and through damaging of β cells, it leads to the inhibition of biosynthesis and insulin secretion in the body, as a result of blood sugar increase.² In line with the above-mentioned contents, our data showed that the FBS level was significantly higher in diabetic mice (Group B) than in the healthy control group (Group A); the difference was significant ($p < 0.05$). However, the FBS level was lower in diabetic mice treated with curcumin (Group C) than in the untreated diabetic group (Group B), which was significant ($p < 0.05$). The interesting point is that the FBS level in diabetic mice treated with curcumin was reduced to almost the same amount of the healthy control group (Figure 2).

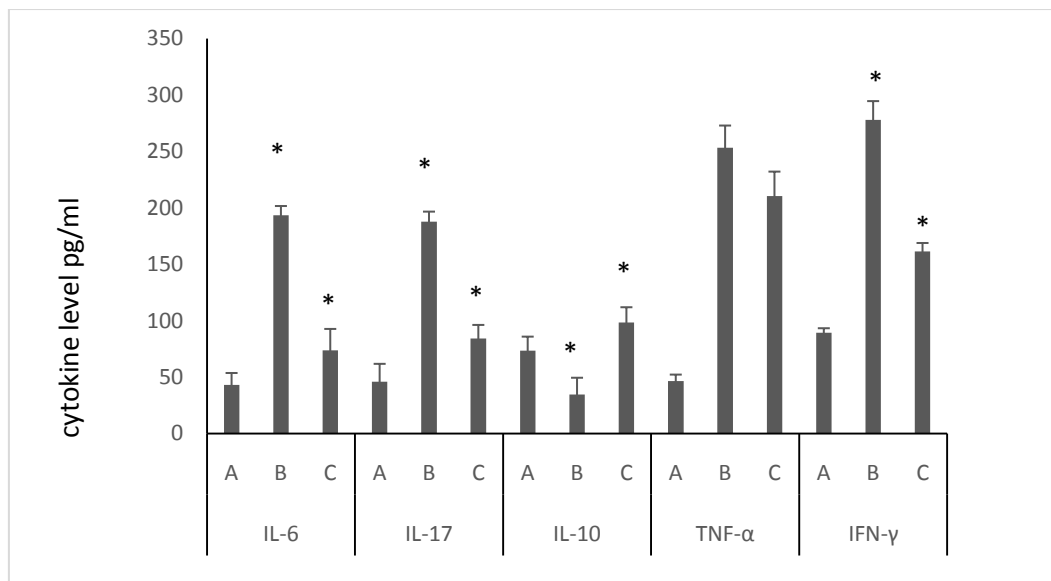


Figure 1. Cytokine concentration in 72 h cell culture supernatant of the spleen in the presence of the pancreatic extract as a stimulator antigen. The control group (A), untreated diabetic group (B), and curcumin-treated group (C); the values are shown as mean \pm SEM. * ($p < 0.05$).

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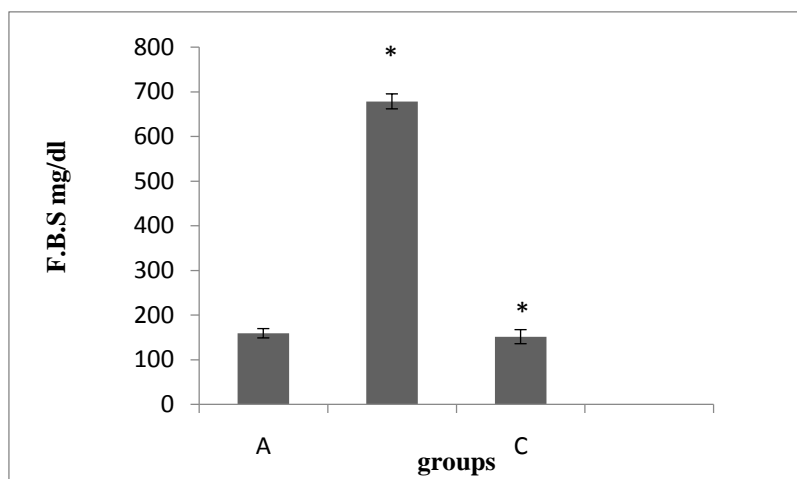


Figure2: Fasting Blood Sugar level in C57BL/6 mice. One day after the last injected dose of curcumin. The control group (A), untreated diabetic group (B), and curcumin-treated group(C); the values are shown as mean±SEM.* ($p<0.05$).

Plasma C-peptide and Insulin Measuring

Plasma C-peptide and insulin are the other important factors in type 1 diabetes which are decreased. At the onset of T1D, the function of the remaining β cells is evaluated by the secretion of C-peptide. This peptide is also an excellent indicator to investigate the expansion of β cells destruction, in addition to reducing endogenous insulin, the decrease in C-peptide levels occurs in the plasma of T1D patients due to the destruction of β cells.^{7,8} As our results indicated, plasma C-peptide levels and insulin

in diabetic mice (Group B) were significantly lower than those of the healthy control group (Group A), which were significant ($p<0.05$). However, plasma C-peptide levels and insulin were higher in diabetic mice treated with curcumin (group C) than in the untreated diabetic group, which were significant ($p<0.05$). These values were significantly higher in diabetic mice treated with curcumin than in the healthy control group, but the difference was not significant ($p<0.05$) (Figure 3).

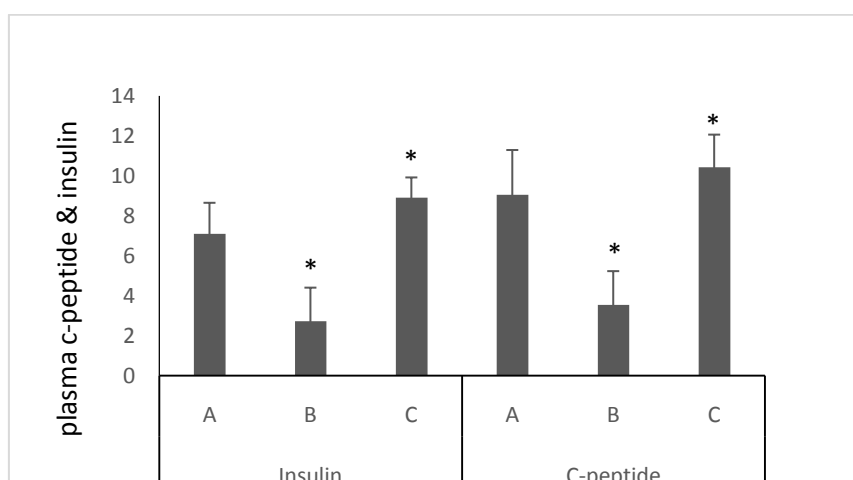


Figure 3: Plasma C-peptide & insulin levels in C57BL/6 mice. One day after the last injected dose of curcumin. The control group (A), untreated diabetic group (B), and curcumin-treated group(C); the values are shown as mean±SEM.* ($p<0.05$).

DISCUSSION

In this study, it appears that curcumin, by decreasing the expression of inflammatory cytokines, causes anti-inflammatory effects and reduces the destruction of cells β in the pancreas Langerhans islets, and consequently it increases insulin and c-peptide levels, there by decreasing FBS. The findings of this study showed a significant reduction in the concentration of inflammatory cytokines in the supernatant of stimulated splenocytes, as well as a decrease in FBS and an increase in plasma insulin and C peptide after 2 weeks of injection of curcumin. These findings support the positive effects of curcumin as an anti-diabetic and anti-inflammatory substance.

Many studies have noted the important role of NF- κ B in the destruction and function of β cells in T1D. It has been revealed that the activation of NF- κ B is induced by some cytokines such as IFN- γ , IL17, TNF- α , and IL-6.¹⁹ Therefore, reduction in inflammatory cytokines or immunosuppressive drugs is a possible means of preventing β cell failure.

Changes in proinflammatory cytokine levels (especially IL-17, TNF- α , IFN- γ , and IL-6) in patients with T1D demonstrate its importance as a potential pathogenes is indicator and the crucial role of these cytokines in promoting complications and problems caused by autoimmune diabetes.²⁰ In line with our results, Rabinovitch et al showed that T helper 1 cells produced by pro-inflammatory cytokines (TNF- α , IFN- γ , IL-1 β , IL-6, and IL-12) activated cytotoxic macrophages and cytotoxic T cells to remove β cell. While anti-inflammatory cytokines (IL-4 and IL-10) produced by activated T helper 2 cells prevented the inflammation of acute destructive pancreatic β cells.⁵ Furthermore, Pennline KJ et al indicated that IL-10 prevented diabetes in mice.²¹ IFN- γ and IL17, along with TNF- α and IL-6, are other cytokines considered to be the main factors creating inflammation in this autoimmune disease²² and inducing the destruction of β cells in the Langerhans islets of the pancreas by accelerating their apoptosis.²³ The present study revealed that the drug reduced IL-6, IL-17, TNF- α , and IFN- γ levels and increased IL-10 levels in the supernatant of stimulated splenocytes of mice treated with curcumin compared to untreated diabetic mice. These changes were significant except in TNF- α ($p < 0.05$).

Meghana et al showed that curcumin could regulate

insulin by regenerating β cells in the Langerhans islets.²⁴ Moreover, Abdel Aziz et al indicated that the induction of diabetes by STZ led to a significant increase in plasma glucose and a significant decrease in plasma insulin and C-peptide levels compared to base levels in mice with T1D. They also demonstrated that oral administration of curcumin in these mice decreased plasma glucose and significantly increased plasma insulin and C-peptide compared to diabetic mice which are consistent with the present study results.²⁵ One study by Chougala et al on STZ-induced diabetic mice showed that oral curcumin was effective in reducing FBS, urinary sugar, and volume.²⁶ Morgan et al also showed that curcumin led to lower blood sugar and a significant increase in plasma insulin.²⁷ In diabetic mice models induced by STZ and diabetic mice induced by alloxan, and in diabetic mice models induced by STZ-nicotinamide,²⁸ oral administration of various doses of curcumin was able to reduce glucose levels and improve insulin sensitivity. In addition, in another study, oral administration of curcumin resulted in a significant reduction in blood sugar in the STZ-induced diabetes model in mice is consistent with the present study results.²⁹ In another study investigating the effects of improving curcumin on diabetes autoimmune disease, Castro et al found that treatment with curcumin resulted in a significant delay in the onset of the disease. In some cases, it prevents autoimmune diabetes by inhibiting leukocyte infiltration and maintaining insulin-producing cells.

Kanitkar et al (2008) in one in vitro study showed that cells in the pancreas islets of C57BL/6 mice treated with curcumin exposed to TNF- α , IL-1 β , and IFN- γ cytokines were protected from cytokine death in vitro. The same researchers in an in vivo study found that the concentration of pro-inflammatory cytokines in the serum and pancreas of mice with autoimmune diabetes was increased, but in mice treated with curcumin before STZ induction, the increase was not observed; these findings are similar to the results found in the present study.³⁰

Furthermore, Abdollahi et al reported that curcumin reduced the expression of Th1 cytokines (IFN- γ , TNF- α , IL-1) and increased the expression of Th2 (IL-4 and IL-10) cytokines in the colon mucosa. It has also been demonstrated that this drug increases the ratio of IFN- γ to IL-4 in the spleen and blood circulation. Some evidence suggests that curcumin, by reducing the expression of IL-6, IL-21, IL-17, as well as inhibiting

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phosphorylation of some transcription factors, inhibits the differentiation and development of IL-17 cells and increases the expression of IL-10 in CNS and lymphatic organs.³¹ Another study found that in mice with deficiency in the IL-10 gene, curcumin could improve the complications of autoimmune colitis.

Prophylactic use of curcumin, owing to its antioxidant properties and the ability of this substance in harvesting free radicals, prevents damage to cells β .²⁴ In short, curcumin can reduce the severity of autoimmune diseases like IBD (inflammatory bowel disease) by reducing the inflammation caused by intrinsic immunity (receptors, cells, cytokines, chemokines). In acquired immunity, boosting Th2 responses (producing IL-4) and reducing adverse Th1 responses (producing IFN- γ) can reduce inflammatory diseases such as IBD and autoimmune diabetes.³¹

However, more studies are needed to confirm the role of curcumin in reducing the complications of autoimmune T1D as well as the relationship between the dose and the response.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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