ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
December 2018; 17(6):564-573.

Carbenoxolone Could Deteriorate Streptozotocin-induced Diabetes through Induction of Heat Shock Protein 70 and IFN-γ in C57BL/6 Mice

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Received: 7 November 2017; Received in revised form: 1 July 2018; Accepted: 8 July 2018

ABSTRACT

Type 1 diabetes (T1D), a spontaneous autoimmune disease, is associated with destruction of insulin-producing β-cells in the pancreas. Since some heat shock proteins (HSP), such as HSP70 exert a protective effect in both tissues and cells, the present study was conducted to elucidate the effects of carbenoxolone (CBX) as an HSP70 inducer on T1D.

The disease was induced in male C57BL/6 mice using streptozotocin (STZ) and subjects were allocated to therapeutic 1 and therapeutic 2 groups, as well as negative and positive control groups. The treated mice (therapeutic 1 and therapeutic 2 groups) received 50 mg/kg CBX intraperitoneally every 24 hours, in the therapeutic 1 group the drug was injected before and after disease induction whereas in the therapeutic 2 group the drug was injected only after disease induction. Serum fasting blood sugar (FBS) level, cytokines production (Interferon-gamma (IFN-γ), Interleukin 10 (IL-10), and IL-17), serum HSP70 level and $CD4^+CD25^+Foxp3^+$ regulatory T cell (Treg) frequency measurements were outperformed 14 days after the last STZ injection.

Our results showed that in the treated groups, serum HSP70, IFN-γ, and IL-17 levels were increased in contrast to the untreated groups. The IL-10 level was markedly decreased in comparison to untreated diabetic mice ($p<0.05$). Moreover, it was found that the frequency of Tregs in treated mice was lower in comparison to the untreated mice but the difference was not significant ($p>0.05$).

Our results confirm that CBX might through HSP70 induction, followed by increasing IFN-γ level leads to suppression of IL-10 production in diabetic mice resulted in toxic effects on pancreatic islet beta cells and deteriorating of disease.

Keywords: Carbenoxolone; Heat shock protein 70; Interleukin-10; Interleukin-17; Interferon-gamma; Mice; Regulatory T cells; Streptozotocin; Type 1 diabetes

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Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

INTRODUCTION

Type I diabetes (T1D) is an autoimmune disease in which the adventitious immune response creates against the insulin-producing beta cells in pancreatic islets that cause cell damage and impaired glucose regulation.[1] T helper type 1 (Th1) and Th17 cells are effectively involved in the pathogenesis of T1D.[2-4] some studies have suggested that inflammation in the early stages of the disease may be due to the induction of producing Th1 cells cytokines such as Interferon-gamma (IFN-γ)[5,6] and recent studies indicated that secreted interleukin 17 (IL-17) by Th17 cells have an important role in comparison with other cytokines in autoimmune diseases such as T1D.[7] Shi and colleagues showed that T1D in mice is caused by an imbalance between $CD4^+CD25^+foxp3^+$ regulatory T cells (Treg cells) and IL-17 producing Th17 cells. In other words, immune tolerance can be achieved by proper and efficient regulation of Treg cells and performance T cells.[8,9] Generally, the balance between Th1/Th17 and Treg/IL-10 secreting cells is critical in determining the immunopathological outcome.[4] Heat shock proteins (HSP) are proteins produced under conditions of pathophysiologic stress and have many protecting roles under stress. HSPs such as HSP70 can induce anti-inflammatory Treg cells that suppress the autoimmune diseases.[10] HSP70 has a potential protective effect on cells and tissues in T1D, but it seems that protective properties of HSPs are impaired.[11] Although HSPs have beneficial effects, their excessive increase can disrupt the cell cycle and cause adverse effects on the cells.[12] On the other hand, it was found that endogenous HSP70 can activate monocytes, induction of intracellular calcium, activation of nuclear factor of kappa B (NF-κB) and pro-inflammatory cytokines secretion of IL-1β, IL-6, and Tumor necrosis factor alpha (TNF-α)[13] (these cytokines are effective in the pathogenesis of T1D)[14-16], also It is Shown that HSP70 leads to increase IFN-γ.[17,18] Carbenoxolone (CBX) is semi-synthetic drug derived from Glycyrrhetinic acid, and was introduced as an anti-inflammatory in 1960 due to structural similarity with steroids.[19,20] This drug is an inducer of HSP, such as HSP 70.[12,21-23] In this study, at different stages of the disease process, we examined the effects of the drug on the FBS, HSP70, the frequency of Tregs and IL-10, IFN-γ, and IL-17 cytokines in C57BL/6 Mice.

MATERIALS AND METHODS

Mouse

Male C57BL/6 mice, 6-8 weeks of age, were purchased from the Pasteur Institute of Iran. Mice were randomly allocated into 4 groups; each group consisted of 10 mice. Group A (negative control) consisted of healthy mice, which received only citrate buffer with pH=4.5. Group B (positive control) consisted of diabetic mice with no treatment. Group C (therapeutic 1) consisted of mice, which 50 mg/kg CBX (Sigma, Germany) was injected intraperitoneally[24,25] 48 hours before and after induction of the disease (two doses before and five doses after induction every 24 hours). Group D (therapeutic 2) mice which was received 50 mg/kg CBX after induction of the disease (five doses, every 24 hours). Five mice of each group were sacrificed by cervical dislocation in order to perform the necessary tests on 14 day after the induction of the disease. Each group of mice were kept in isolated cages and clean room with a constant temperature of 25°C and 12-hour cycle of light and darkness and had access to adequate food and water. This study has been approved in ethics committee of Tabriz University, No. j-325/6 (2015.04.15).

Diabetic Mice Induction

Before administering each dose of Streptozotocin (STZ) (Sigma, Germany), mice were fasted for 4 hours. They received STZ intraperitoneally for 5 days (50 mg/kg in 200 micro liters of citrate buffer with pH=4.5, which was prepared ten minutes before the administration).[26]

Streptozotocin is a natural product that is toxic to pancreatic insulin-producing beta cells and widely used to induction of T1D in mice.[27] For FBS level evaluating, blood was collected from the tail vein of mice by insulin syringes and then blood glucose level was measured 14 days after the last STZ injection by the automatic glucometer (ACCU-CHEK Compact plus, Ireland) (before an assessment, all groups were fasted for 4 hours). As previously reported, diabetic mice were confirmed histopathologically using hematoxylin-eosin staining of paraffin-embedded tissue sections.[28]

Pancreatic Extracts

In order to prepare pancreatic extracts as irritating antigen for spleen cells five mice were sacrificed by

cervical dislocation and their pancreas were collected immediately and were homogenized in cold Phosphate-buffered saline containing protease inhibitor (Sigma Co, Germany) after weighing, Homogenized tissue was placed in two stages in a centrifuge, the first phase was 3000 rpm for 10 min and the second phase was 12,000 rpm for 20 minutes at 4°C. The supernatant that was clear extracts were collected. Bradford method was used to measure protein concentration of the extract and extract protein concentrations were measured.[29]

Spleen Cell Culture

In order to cultivate the spleen cells and use of cell culture supernatant for Cytokines measuring five mice of each group were sacrificed 2 weeks after the final dose of STZ, their spleens were collected under sterile conditions and were transferred to Petri dishes containing 5ml medium (Sigma America) RPMI-1640 containing 10% albumin bovine serum (FBS) (Gibco German company). Samples were completely cut into small pieces and obtained tissue was passed through wire mesh with 1.0 mm diameter to prepare cell suspension and removal of excess tissue blocks. The obtained cell suspension (3 mL) was placed in pipes centrifuges and 3ml ficoll (Sigma, Germany) was slowly added to it so that ficoll was placed below the cell suspension and followed by 15 minutes at 4°C in 600g and centrifuged. The obtained cell pellet was washed two times by PBS and were cultured in 24 houses plate (the number of 2×10^6 cell/ml from each sample) containing RPMI-1640 medium enriched by 10% FBS in the presence and absence of pancreas extract as irritating antigen with concentration of 50 µg/mL for 72 hours in an incubator containing 5% CO2.[30]

Cytokines Measuring in Cell Culture Supernatant

Cell culture supernatant was collected after 72h, and the presence of IL-10, IL-17 and IFN-γ were studied. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used (Bender Med Co., Austria). The concentrations of cytokines were expressed as average (pg/mL) and standard curve was depicted between 15.6-1000 pg/mL (IL-10, IFN-γ) and 7.8-500 pg/mL (IL-17) of cytokine in the solution. The sensitivity of test was 5.0 pg/ ml for IL-10, 1.6 pg/ mL for IL-17 and 5.3 pg/ mL for IFN-γ. The results of four subjects were expressed as mean±SEM.

Flow Cytometric Analysis

Tregs frequency was evaluated 14 days after the final STZ administration using mouse Treg cell staining kit (Ebioscience Co., UK) and flow cytometry (Partec, Germany). According to the manufacturer's protocol, the cell suspensions (1×10^6 cells) were prepared from spleens of C57BL/6 mice and were washed and re-suspended in staining buffer. The Frequency of Tregs was determined by 3-color flow cytometry using monoclonal antibodies of (FITC) – conjugated anti-mouse $CD4^+$, Allophycocyanin (APC)-conjugated anti-mouse CD25, phycoerythrin (PE) – conjugated anti-mouse foxp3 and PE-conjugated rat IgG2a isotype control. For staining surface marker, cells were incubated for 30 min at 4°C in the dark with anti-CD4 and anti-CD25 antibodies. The cells were then washed in cold staining buffer, the supernatants were discarded, and the procedure was followed by re-suspending the cells in freshly prepared fixation/permeabilization working solution and incubation for 30 min at 4°C. For intracellular staining, anti–mouse/rat foxp3 (Fjk-16s, ebioscience Co., UK) antibody or isotype control were added in 1x permeabilization buffer and incubated for 30 min at 4°C. Finally, after washing the cells with 1x permeabilization buffer, they were re-suspended in appropriate volume of flow cytometry staining buffer and analyzed with flow cytometer (Partec Co., Germany) and FlowMax software.

Evaluation of HSP 70

Two weeks after the final dose of STZ, mice were sacrificed by cervical dislocation and blood was collected from their hearts by insulin syringe, and their HSP70 protein of their blood serum was measured by ELISA kit (Company of Stress Gen Biotechnologies, America). Standard curve of the protein in solution were drawn 0.2 to 125 ng/mL. Sensitivity of tests was 0.09 ng/mL and HSP70 protein concentration was measured by the standard curve.

Statistical Analysis

The analysis of variance was performed by ANOVA test and mean comparison were performed using Tukey HSD by SPSS version 19.0. In all studies, the value ($p<0.05$) was considered as a significant level. All data were expressed as mean±SEM.

RESULTS

FBS level measurement

Measurement of fasting blood sugar level, 14 days after the last administration of streptozotocin, showed that FBS level in mice which were received CBX (group C and D) was significantly higher than the untreated diabetic group (B) (p<0.05) and this difference in group C mice that received the drug before and after the onset of the disease was higher than the other groups (Figure1).

IL-10, IL-17 and IFN-γ Assay

As previously mentioned, the balance between Th1/Th17 and Treg cells is critical in determining the immunopathological outcome of T1D, resulting in Th1 cytokines such as IFN-γ, Th17 cytokines such as IL-17 and Treg cytokines such as IL-10 in T1D pathogenesis are important. The level of IL-10 in the mice which were received CBX (group C and D) was markedly lower than the untreated diabetic group (B) (p<0.05), but the level of IL-17 in these groups was significantly more than the untreated diabetic group (B) (p<0.05). IFN-γ, in the mice which were received CBX (group C and D) was markedly higher than the untreated diabetic group (B) (p<0.05) (Figure 2).

Serum Level of HSP 70

As previously mentioned, HSP70 can induce Treg cells that suppress the autoimmune diseases, as well as HSP70 has a potential protective effect on cells and tissues in T1D. Our results show compared to the diabetic mice without treatment (group B) mean levels of serum HSP70 in diabetic mice treated with CBX (group C and D) was significantly higher (p<0.05). Furthermore, serum HSP70 mean level was much high in group C (Figure 3).

Tregs Frequency

Since Treg cells secrete IL-10, we used this factor to find out the effects and frequency changes of Treg cells, but since the Treg cells are not the only source of IL-10 production, by the flow cytometric method, We measured the frequency of Treg cells (Figure 4). Flow cytometry analysis suggested that in comparison to the untreated diabetic mice (group B), expansion of Tregs in CBX treated groups (group C and D) was higher but difference was not significant (p>0.05) (Figure 5).

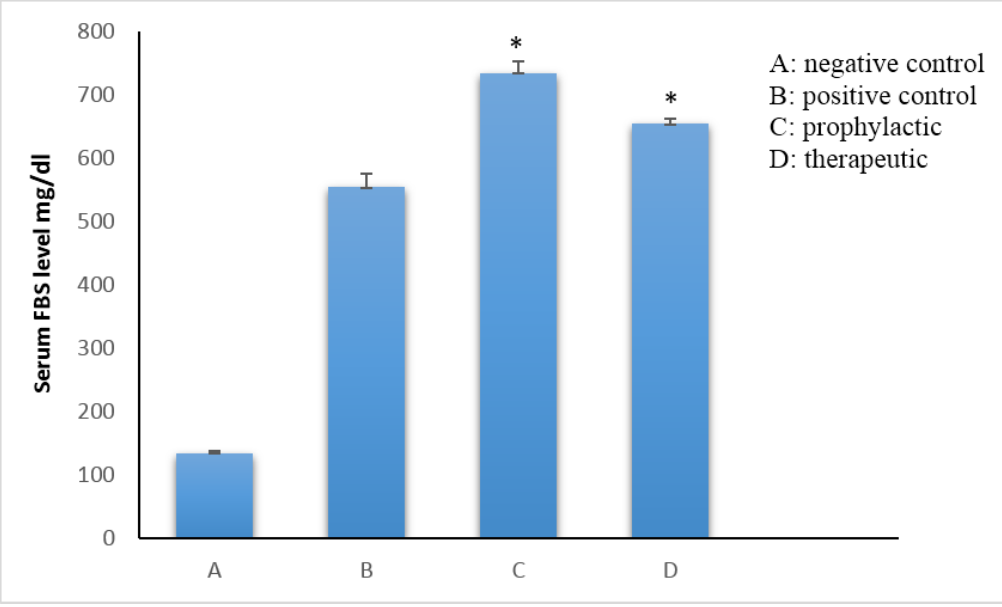

Figure 1. Serum fasting blood sugar (FBS) level on 14th day after the final dose of streptozotocin administration in mice
*** Indicates a significant difference at the level (p<0.05) between groups receiving the drug and untreated diabetic group**

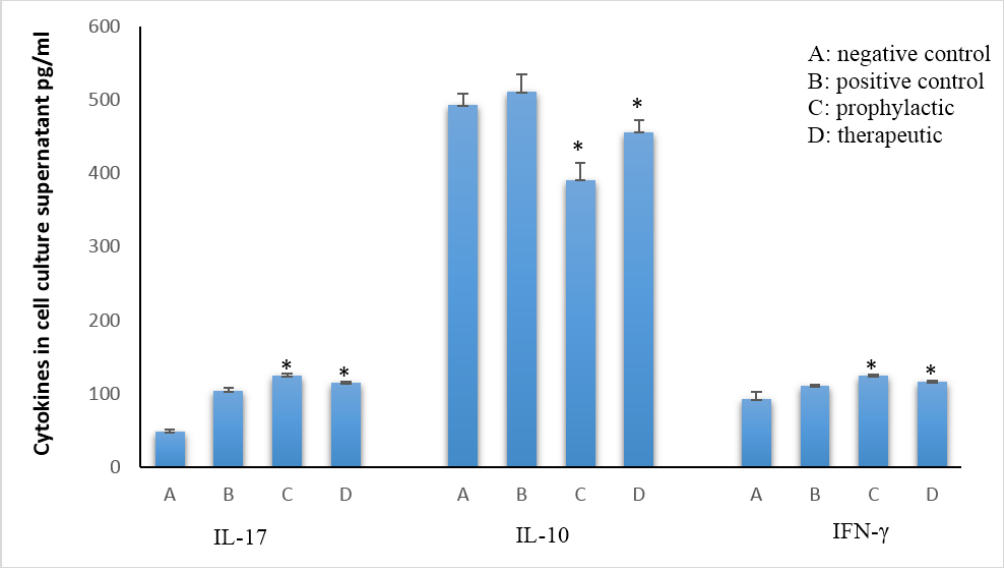

Figure 2. Interlukin 17 (IL-17), IL-10 and interferon-gamma (IFN-γ) in the culture supernatants of spleen cells after 72 hours
Since IFN-γ, IL-10 and IL-17 cytokines have an important role in type 1 diabetes, the presence of these cytokines was studied. For this purpose, the spleen cells of mice were incubated for 72 hours in the presence of pancreatic extract as irritating antigen and then cell culture supernatant was used to measure cytokines by ELISA.
*** Indicates a significant difference at the level (p<0.05) between groups receiving the drug and untreated diabetic group**

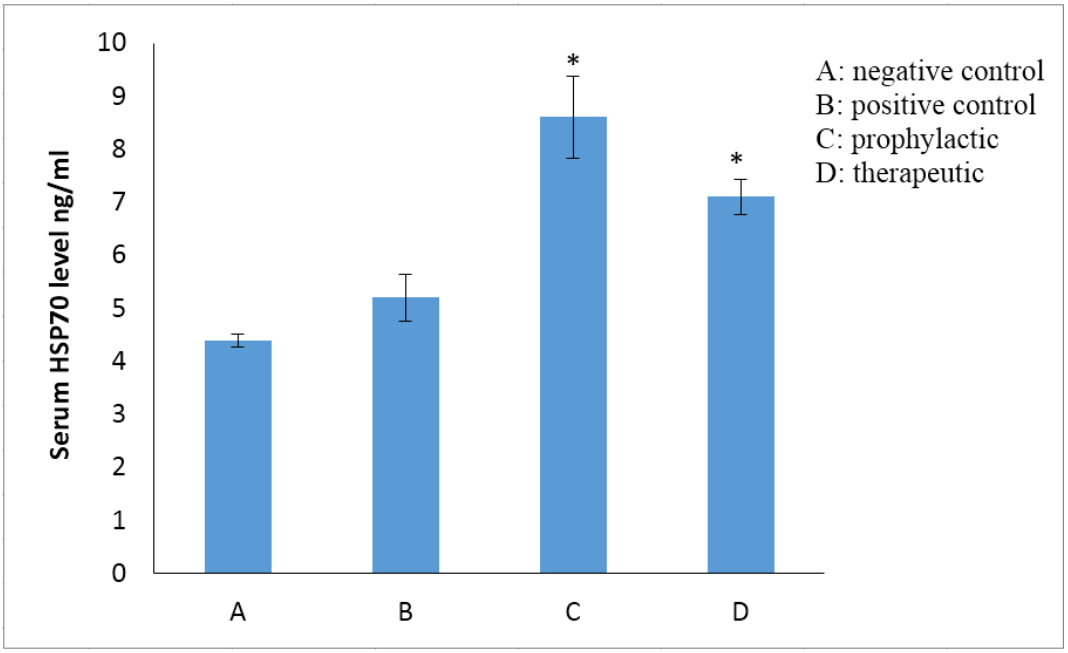

Figure 3. Serum heat shock protein 70 (HSP70) level on 14 days after the final dose of stereptozotocin administration in mice
Since HSP70 has a potent protective effect on cells and tissues in type 1 diabetes and carbenoxolone was used as an inducer of HSP70, serum HSP70 levels were measured using ELISA.
*** Indicates a significant difference at the level (p <0.05) between groups receiving the drug and untreated diabetic group**

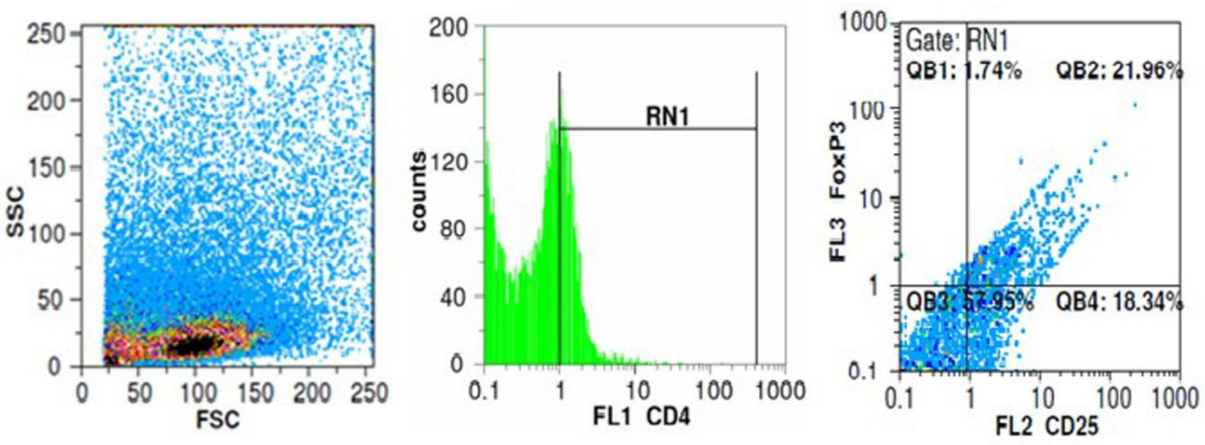

Figure 4. The results of flow cytometric analysis of regulatory T cells (Treg) in cell suspensions prepared from spleens of mice
Since Treg cells secrete IL-10, we used this factor to find out the effects and frequency changes of Treg cells, but since the Treg cells are not the only source of IL-10 production, by the flow cytometric method, the frequency of Treg cells was measured.
The isolated splenic cells were stained according to manufacturer's instructions and analyzed by flow cytometry. At first $CD4^+$ lymphocytes was shown in gate RN1 then $CD25^+Foxp3^+$ cells were identified in the same gate (QB2).

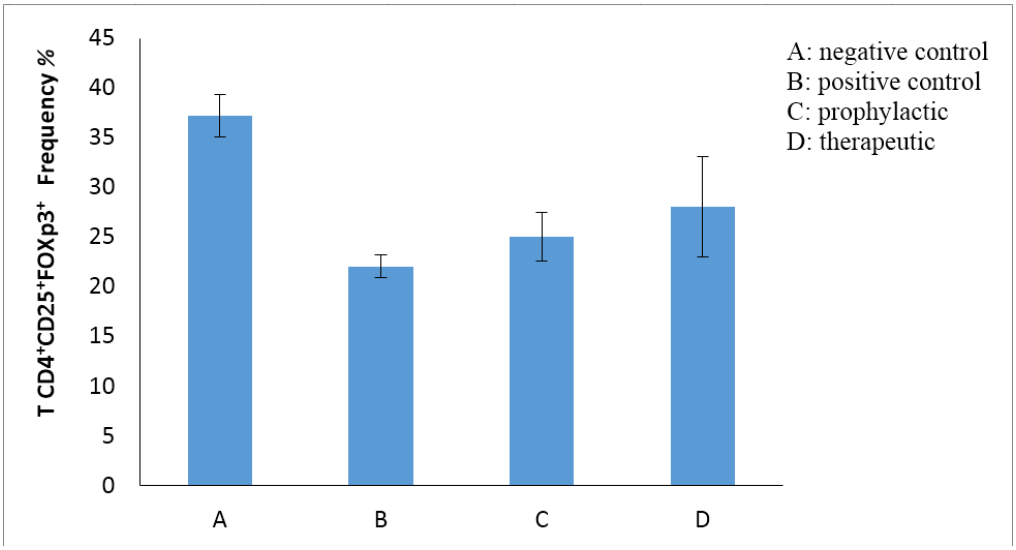

Figure 5. Percentage of regulatory T cell (Treg) frequency in cell suspensions prepared from spleens of mice
Since Treg cells secrete IL-10, we used this factor to find out the effects and frequency changes of Treg cells, but since the Treg cells are not the only source of IL-10 production, by the flow cytometric method, the frequency of Treg cells was measured.
(The Results are expressed as mean ± SEM)

DISCUSSION

The present study was conducted to clarify effects of CBX as an HSP70 inducer in autoimmune diabetes in C57BL/6 mice. The results show that serum HSP70 in therapeutic 1 (receiving seven doses of the drug) and therapeutic 2 (receiving five doses of the drug) mice which received CBX in comparison to the untreated mice significantly increased (p<0.05) and in therapeutic 1 mice it was markedly higher than therapeutic 2 mice (p<0.05). The results of serum FBS level showed that FBS level in treated diabetic mice (therapeutic 1 and therapeutic 2 groups) was significantly higher than the untreated diabetic mice (p<0.05). In accordance with our observation, CBX might have toxic effects through increased levels of pro-inflammatory cytokines which were occurred followed by excessive serum HSP70 induction. Although HSPs have beneficial effects, their excessive increase can disrupt the cell cycle and cause adverse effects on the cells.[12] Various studies have shown that CBX can cause toxic effects, Laubenthal et al. in a study showed that CBX can cause toxic effects by interfering with mitochondrial bioenergetic activity.[31] Jazani Hosseini et al that studied different doses of CBX to reduce inflammation caused by salmonella enterica lipopolysaccharide in the mouse, their results showed that 20 mg/kg of CBX caused lowest mortality rate in mice, and higher doses of that increased toxicity and mortality in mice.[31] On the other hand, Lee et al showed that CBX at high concentrations (50-100 µmol per liter) has significant cell toxic effects on RAW 264.7 cells.[32] Our results about cytokines showed that IFN-γ and Il-17 levels in the treated mice with CBX were significantly higher than untreated diabetic mice (p<0.05) and between the two treated groups, the level of these cytokines in the therapeutic 1 group was higher than the other (p<0.05). However, IL-10 level in the treated diabetic mice was significantly lower than the untreated diabetic mice (p<0.05). A study by Abbey et al. showed that glycyrrhizin could increase IFN-γ levels in serum, which CBX is derived from it[33] and various studies have shown that HSP70 increased IFN- γ.[17,18] As a hypothesis, we propose that CBX through the HSP70 induction increases IFN-γ levels and subsequently, leads to toxic effects. The studies of Jacobs et al. demonstrate that in vivo administration of IFN-γ has been worsening autoimmune diseases in NZB/W F1 mice, while administration of anti-IFN-γ has been shown to improve disease.[34] IFN-γ inhibits the growth of Th2 population, increases the presentation and processing of antigen and costimulatory molecules on antigen presenting cells, thereby leading to an increase in the cytotoxic immunity.[35] It has been demonstrated that IFN-γ could suppress Th2 cells via induction of Th1 cells differentiation.[36] Several studies have also shown that IFN-γ inhibits release of IL-10 anti-inflammatory cytokine.[37-40] In a survey which Schloot et al conducted, they found out that IFN-γ in comparison to IL-10 in Non-obese diabetic (NOD) mice was more than non-diabetic mice.[41] Th1 cells that secret IFN-γ are major mediators of own responses in pancreatic beta cells, while Th2 cells could cause protection against autoimmune diabetes by secreting IL-10.[42] It seems that CBX could increase the disease severity by increasing production of IFN-γ followed by, suppressing production of IL-10. Similar to our study, results showed that administration of cyclophosphamide in C57BL/6 KsJ mice, after induction of insulin-dependent diabetes with STZ, could aggravate the disease because of Th0 cells polarization to Th1 cells, which has been caused systemic cytokines shifts to the production of IFN-γ.[43] Our results about Treg cells frequency show that this frequency in treated and untreated groups has some differences but not significant. IL-10 secreting Treg cells that are seen in people with T1D are associated with less invasive autoimmunity, as confirmed by a reduced number of pro-inflammatory Islet-specific T cells and lower levels of autoantibodies.[44] Studies have shown that naive T $CD4^+$ cells differentiation to Th17 cells or Treg cells depends on the type of cytokines in the environment.[7] Transforming growth factor-β (TGF-β), induces production of Tregs, while TGF- β along with IL-6 inhibited Treg cells production and leads to the production of pro-inflammatory Th17 cells.[3,7,45] The Balance between Treg cells and Th17 cells is controlled by IL-6[46] and the frequency of Treg cells and Th17 cells are inversely related together.[45] Recent studies in murine collagen-induced arthritis model showed that neutralizing antibody against the IL-6R significantly reduced the disease score by reducing the percentage of Th17 cells and increasing the percentage of Treg cells.[47] A study has shown that released HSP70 from myeloma cells leads to increase the secretion of IL-6 from stromal cells.[48] As well as other research has shown that exogenous HSP70 increases the IL-6 by affecting monocytes by activating the pathway

dependent on CD14 and intracellular calcium.[49] Also, in a study, results showed that endogenous HSP70 could activate monocytes, induce intracellular calcium, activate NF-kB and produce pro-inflammatory cytokines IL-1β, IL-6, and TNF-α.[13] As a hypothesis, we suggest that CBX through induction of HSP70 production has caused increasing pro-inflammatory cytokines such as IL-6 and this cytokine has increased the production of IL-17–producing Th17 cells and has suppressed the population of Treg cells. Th17 cells, by producing pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α, and chemokines, promote inflammation and mobilize Th1 cells into target tissues. Since pre-inflammatory cytokines such as IL-6, which protect performance T cells from the suppression effect of Treg cells, are present in large amounts, Treg cell accumulation is not effective in target tissues. Therefore, increasing the frequency of Treg cells along with reduced inflammation of the tissue can be an effective strategy for the treatment of autoimmune diseases, such as T1D.[50] It should be noted that this study has some limitations. Firstly, since in the present study there were very few references available to us, one of our most important problems was the finding of right drug dosage which it was mentioned in fewer articles, not so completely. Secondly, managing the use of two important drugs, each of which could cause the death of mice in case of inaccurate drug administration. Tertiary, although we worked in vivo conditions, it was very critical to observe sterile conditions in the cultivation of mice spleen cells with Pancreatic extract as Antigen. In conclusion, in this study, it could be established that CBX through the induction of HSP70 increased levels of IFN-γ and IL-6. Then IFN-γ via induction of Th1 cells differentiation suppressed IL-10 producing T cells. On the other hand, IL-6 increased Th17 cells population and this Th17 cells along with pro-inflammatory cytokines such as IL-6 have led to the recruitment of Th1 cells to the target tissue. In summary, the present study showed that CBX by induction of excessive hsp70 increased the level of IFN-γ and IL-17 and decreased the level of IL-10. Then serum FBS level increased and STZ-induced diabetes in C57BL6 Mice has deteriorated.

ACKNOWLEDGEMENT

We would like to express our thanks to the university of Tabriz and faculty of veterinary medicine for financial support.

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