BORON PROMOTES THE SURVIVAL AND THE MAINTENANCE OF β-CELLS IN TYPE I DIABETES MELLITUS

by
Safa Aydın

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APPROVED BY:

Prof. Dr. Fikrettin Şahin (Thesis Supervisor) .....

Prof. Dr. Ertuğrul Kılıç .....

Assoc. Prof. Dr. Dilek Telci .....

Assoc. Prof. Dr. Ferruh Özcan .....

Assist. Prof. Dr. Hüseyin Çimen .....

DATE OF APPROVAL: ..../..../2016

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ABSTRACT

BORON PROMOTES THE SURVIVAL AND THE MAINTENANCE OF β-CELLS IN TYPE I DIABETES MELLITUS

Diabetes mellitus is worldwide disease. The life of diabetic patients are dependent on exogeneous insulin. Pancreas or particularly islet transplantations are performed for treatment to heal this disease. External substances are also used to achieve improvements in the healing process of the disease.

In the current study, two different boron derivatives (boric acid-BA and sodium pentaborate pentahydrate-NaB) were investigated for their effect on pancreatic cells in terms of pro-apoptotic and anti-apoptotic markers, genes related to insulin production mechanism, pancreatic development and glucose metabolism, some antioxidant enzymes, and genes for the initiation of diabetes, insulin secretion and antioxidant enzyme activities *in vitro*. The results revealed that boron derivatives did not lead to apoptosis. On the contrary, they increased cell viability, antioxidant enzyme activities and insulin secretion in the gene level.

Overall evaluation, data in the current study showed that boron derivatives might be promising therapeutic agents for type I diabetes. However, additional investigations are strictly needed to elucidate molecular mechanisms of boron derivatives.

ÖZET

BOR TİP 1 ŞEKER HASTALIĞINDAKİ β HÜCRELERİNİN HAYATTA KALMASINI VE CANLILIĞINI SÜRDÜRMESİNİ TETİKLER

Diyabet dünya çapında bir hastalıktır. Diyabet hastalığına sahip insanlar dış kaynaklı insulin bağımlıdırlar. Hastalığı iyileştirmek için pancreas yada özel olarak adacık nakli yapılır. Hastalığın gelişim sürecinde iyileştirmeler elde etmek için harici maddeler de kullanılmaktadır.

Söz konusu çalışmada, boron türevlerinin *in vitro* koşullardaki (borik asit-BA ve sodyum pentaborat pentahidrat-NaB) proapoptoptik ve antiapoptotik, insülin üretimi, pankreatik gelişim ve glikoz metabolizması, bazı antioksidan enzimler ve diyabetin gelişimi üzerine koruyucu rolleri olan genler ile insülin salınımı ve antioksidan enzim aktiviteleri incelenmiştir. Sonuçlar boron türevlerinin apoptoza yol açmadığını ortaya koymuştur. Buna ek olarak, hücre canlılığını, antioksidan enzim aktivitelerini ve insülin salınımını gen düzeyinde arttırmışlardır.

Bu çalışmadan toplanan veriler, boron türevlerinin tip 1 diyabet için potansiyel umut verici terapötik ajanlar olduklarını ortaya çıkarmıştır. Ancak, boron türevlerinin moleküler mekanizmalarının açıklanması için ek araştırmalara şiddetle ihtiyaç duyulmaktadır.

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LIST OF SYMBOLS/ABBREVIATIONS

AGEs	Advanced glycation endproducts
B	Boron
BA	Boric Acid
BB	Biobreeding
CAT	Catalase and
cDNA	Complementary DNA
CO_2	Carbon dioxide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DU	145 human prostate cancer cell line
ECM	Extra Cellular Matrix
ELISA	Enzyme-linked immunosorbent assay
Fas L	Fas Ligand
FasR	The Fas receptor
FBS	Fetal bovine serum
FPGT	Fasting plasma glucose test
FR167653	p38 mitogen-activated protein kinase inhibitor
GAD_{65}	Glutamic acid decarboxylase
G-CSF	Granulocyte colony stimulating factor
Gck	Glucokinase
GDM	Gestational diabetes mellitus
GDP	Glyceraldehyde 3-phosphate dehydrogenase
GFAT	Glutamine:fructose-6-phosphate amidotransferase

Glut2	Glucose transporter 2
GM-CSF	Granulocyte-macrophage-colony stimulating factor
GPX1	Glutathione peroxidase 1
H_2O	Water
H_2O_2	Hydrogen peroxide
H_3BO_3	Boric Acid
HLA	Human leukocyte antigen
HNF	Hepatocyte nuclear factor
HOCl	Hydrochlorous acid
HRO_2	Hydroperoxyl
HSPs	Heat shock proteins
IA-2	Insulin-associated antigen-2
IAAs	Anti-insulin antibodies
IDDM	Insulin-dependent diabetes mellitus
IFN-γ	Interferon gamma
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-1β	Interleukin-1 Beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6
ISL-1	Insulin gene enhancer protein
JNK	Jun amino-terminal kinases
LDL	Low density lipoprotein
LNCaP	Androgen-sensitive human prostate adenocarcinoma cells
MAPK	Mitogen-activated protein kinase
MHC	Major histocompatibility complex
MODY	Maturity-onset diabetes of the young

mRNA	Messenger RNA
MTS	3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H tetrazolium
NaB	Sodium pentaborate pentahydrate
NaBC1	Na^+ Coupled Borate Transporter
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor kappa B
NIDDM	Non- insulin-dependent diabetes mellitus
NO	Nitric oxide
NOD	Non-Obese Diabetic
O_2	Oxygen
O_2^-	Superoxide
OGTT	Oral glucose tolerance test
OH	Hydroxyl
PBS	Phosphate-buffered saline
PC-3	Human prostate cancer cell line
PDX-1	Pancreatic and duodenal homeobox 1
PI	Propidium iodide
PON	Peroxynitrite
PPI	Preproinsulin
PPP	Pentose Phosphate Pathway
PS	Phosphatidylserine
PSA	Penicillin, streptomycin, and amphotericin B
PTP	The protein tyrosine phosphatase
RAGE	The receptor for advanced glycation endproducts
RAI	Radioimmunoassay
RIN5AH	Rat insulinoma cells
RNA	Ribonucleic acid

RNS	Reactive nitrogen species
RO_2	Peroxyl
ROS	Reactive oxygen species
RT-PCR	Real time polymerase chain reaction
SAPK	Stress-activated protein kinases
SOD	Superoxide dismutase
SOD1	Superoxide dismutase 1
SOD2	Superoxide dismutase 2
STZ	Streptozotocin
T1D	Type 1 diabetes
TGF-β	Transforming growth factor beta
Th2	T hepler 2
Th3	T hepler 3
TNF-α	Tumor necrosis factor alpha
UDP-Glc-NAc	Uridine diphosphate *N*-acetylglucosamine
VEGF	Vascular Endothelial Growth Factor
ZnT8	Zinc transporter 8
β-TC-6 cells	Pancreatic insulinoma cell line

1. INTRODUCTION

Pancreas is an organ located in the upper left abdomen, behind the stomach. It mainly has two functions. Exocrine function which helps in digestion and endocrine function that regulates blood sugar level. Endocrine cells of the pancreas is located in islets of Langerhans. There are four types of endocrine cells. The β cells, which secrete insulin hormone, are accounting for 60-80 per cent of total cell number of endocrine cells, while 15-20 per cent of α cells which secrete glucagon hormone. δ cells, secreting somatostatin hormone, are less than 10 per cent of endocrine cells, while less than one per cent of endocrine cells are PP-cells [262,263,264].

Pancreatic β cells can secrete insulin hormone and glucose is the major nutrition for this process. It was reported that insulin release by glucose stimuli is biphasic and pulsatile [265]. Glucose-stimuli insulin secretion from β cells is a multistep process and highly associated with Ca^{2+} oscillations. Firstly, glucose is transported by glucose transporter mediated by facilitated diffusion. Glucose is then phosphorylated to glucose-6-phosphate by glucokinase enzyme which is the glucosensor of β cells. Glucose undergoes mitochondrial metabolism as well as phosphorylation. As a result of these changes, ADP:ATP ratio increases in the cell. Elevated ADP:ATP proportion mediates depolarization of the cell membrane as well as the closure of ATP-sensitive K_{ATP} channels which induces the opening of voltage-dependent Ca^{2+} channels. Transition of Ca^{2+} triggers the fusion of insulin containing granules with the plasma membrane and insulin is released from β cell [266]. If insulin hormone can not be produced or used by the body, it causes a disease called diabetes.

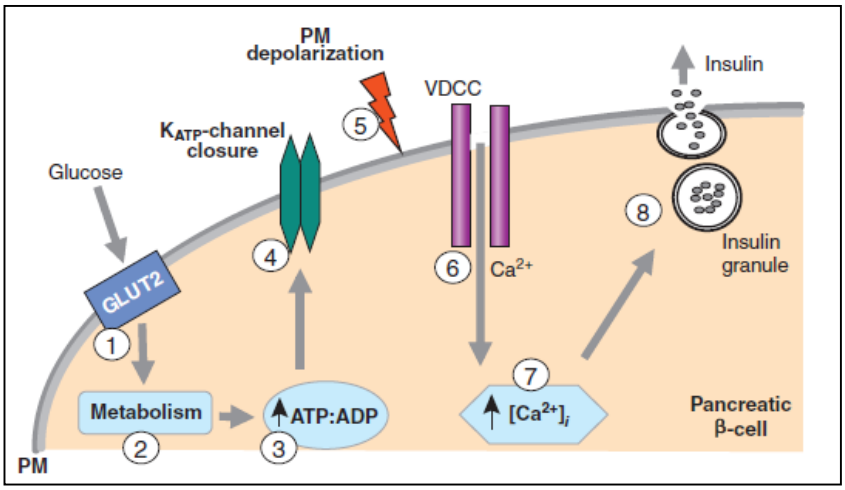

Figure 1.1. The pathway of glucose-stimuli insulin secretion [266]. Abbreviations: PM: Plasma membrane, Glut2: Glucose Transporter 2, VDCC: Voltage-dependent calcium channels, ATP: Adenosine triphosphate, ADP: Adenosine diphosphate

Diabetes mellitus is one of the first diseases in the world. It was firstly described in manuscript belonging to Egyptians. The distinction between type I and type II diabetes, which are basic types of diabetes, was firstly made in 1936[1]. Diabetes mellitus is a disease qualified by hyperglycemia caused by defects in insulin secretion and insulin action or both. Diabetes can affect several systems by causing damage in the human body. Long-term damage, impairment and failure of different organs such as the kidneys, heart and eyes can be expected as the outcomes of diabetes mellitus. It may represent characteristic signs such as thirst, loss of weight, polyuria and defect of vision. Long-term impacts of diabetes mellitus include progressive development of the definite complications of retinopathy, nephropathy that may cause renal failure and neuropathy with risk of amputation. In the subsequent steps of the disease, a non-ketotic hyperosmolar state or even ketoacidosis may develop and lead to stupor, coma and, in absence of effective treatment, death. People with diabetes are at increased risk of cardiovascular and cerebrovascular diseases. There are distinct pathogenic processes which are involved in the development of diabetes. Insufficient function of insulin on target tissues resulting from lack of insulin can cause some abnormalities of protein and carbohydrate metabolism.

The main causes of diabetes are varios. Unhealthy lifestyle factors such as overeating, obesity and physical inactivity can damage the usage of insulin by the body. Furthermore,

uncontrollable risk factors such as age, family history and genetics can also be involved in the development of the disease. In addition, diseases such as pancreatitis can contribute to diabetes.

1.1 TYPES OF DIABETES

Scientists are still working on classification of diabetes and establishing their prevelance in the population. Basically, kinds of diabetes mellitus include type I, type II, other specific types and gestational diabetes.

1.1.1. Type I Diabetes Mellitus

Type I diabetes mellitus is chracterized by the disruption of insulin-producing beta cells of the pancreas by the immune system of the host body[2]. Type I diabetes usually develops more rapidly than other forms of diabetes. It is usually diagnosed in children and occasionally in young adults. The ratio of pancreatic β-cells destruction is varies among individuals [3]. Patients with Type I diabetes must regularly use exogenous insulin (intake) [4]. Type I diabetes are generally referred as juvenile diabetes or insulin-dependent diabetes mellitus (IDDM). However, the term ofjuvenile diabetes is not always accuratebecause adults can also develop type I diabetes. People with type I diabetes also develop insulin resistance resulting from genetics factors and weight gain.

1.1.2. Type II Diabetes Mellitus

Type II diabetes is generally named as non-insulin dependent diabetes which is the most common type of diabetes. It is a metabolic sickness caused by inadequate insulin release and insensitivity of the body to insulin. In these patients, the pancreas produces insulin but the body is not able to use this hormone because of non-insulin dependent diabetes mellitus (NIDDM) and adult-onset diabetes but the term of NIDDM is not always accurate because some patients require insulin treatment. Type II diabetes encompass individuals who have insulin resistance and insulin deficiency. NIDDM can stay hidden for years because hyperglycemia is not always potent enough to induce symptoms of diabetes [5]. Patients

with type II diabetes do not need insulin uptake to survive. There are many situationsleading to type II diabetes mellitus. β-cell destruction, which is specific for type I diabetes does not occur in NIDDM. People with type II diabetes are usually obese and obesity itself causes insulin resistance. Healthy diet may decrease the risk of diabetes development[6]. The most common types of diabetes is type II with a rate of 90-95 per cent of all diabetes [7].

1.1.3. Other Specific Types of Diabetes

Genetic defects of the β-cell: Monogenetic failures in β-cells function can cause several types of diabetes. Metabolic peculiarities related to insulin receptor mutations transfrom hyperinsulinemia to severe diabetes. These mutations may result in acanthosis nigricans, which is hyperpigmentation of the skin, in some individuals[8]. The main characteristic of these types of diabetes is an onset of hyperglycemia at an early age. They are called maturity-onset diabetes of the young (MODY) and are associated with impaired insulin release with minimum or no defects in insulin action[9,10]. As the most common formof genetic defect is associated with mutations on chromosome 12 in a hepatic transcription factor referred to as hepatocyte nuclear factor (HNF) -1α regulated by HNF-4α[11,12], the second form is related to mutations on chromosome 7p in the glucokinase gene results in an inaccurate glukinase molecule. Glucose is turned into glucose-6 phosphate by glucokinase, and glucose-6 phosphate stimulates insulin release from pancreatic β-cells. A third form of monogenic defects in β-cell is associated with a mutation in the HNF4α gene which is located on the chromosome 20q. HNF4α is a transcription factor which takes charge in the regulation of HNF1α [13].

Genetic defects in insulin action: Mutations in insulin receptor may cause abnormalities in insulin function. The metabolic abnormalities related to mutations in insulin receptor may be listed from hyperglycemia to severe diabetes[14]. People with these mutations may develop acanthosis nigricans which is a skin disease. Individuals with The Rabson-Mendenhall and Leprechaunismand syndromes have a mutation in their insulin receptor gene resulting in changes in the function of insulin receptor and insulin resistance[15]

Drug or chemical-induced diabetes: Insulin release may be damaged by lots of drugs. These drugs may not result in diabetes but they may set the stage for diabetes in

individuals with insulin resistance[16]. Some toxin substances such as Vacor and Streptozotocin destroy β-cells of the pancreas[17]. Hormones and medicines can also damage insulin action (i.e. nicotinic acid and glucocorticoids). It has been reported that people receiving α-interferon have developed diabetes associated with islet cell autoantibodies[18].

Infections: It has been shown that certain viruses can have a role in the pathogenesis of pancreatic β-cells. Diabetes can develop in patients with congenital rubella, a syndrome can occur in a developing fetus of a pregnant woman who has contracted rubella virus. Moreover, adenovirus, cytomegalovirus and coxsackievirus B take in charge in inducing basic cases of the disease[8]. Potential manners are molecular similarity and toxic effects of the virus to β-cells on the pancreas. In the patients with type I diabetes, Enterovirus mRNA has been found[19].

1.1.4. Gestational Diabetes Mellitus

Gestational diabetes mellitus (GDM) is a metabolic disorder in which non-diabetic women can develop diabetes during pregnancy. It is described as any degree of glucose intolerance[20]. Women with GDM exhibit high glucose level during pregnancy. Hormonal changes may contribute to this disease as well as family history of diabetes and excess weight[21]. The precise mechanisms of GDM are still unknown. The most distinctive feature of GDM is elevated insulin resistance. Pregnancy hormones and other factors such as excess weight are thought to interfere with insulin action. Insulin normally enhances the entry of glucose into cells. However, insulin resistance inhibits glucose uptake by the cells. Eventually, glucose remains in the bloodstream where glucose level increases. Women with GDM need more insulin to overcome this resistance; about 1,5- 2,5 fold more insulin is produced than in a normal pregnancy. Notably, women with GDM are tend to obesity, type 2 diabetes mellitus and some metabolic syndromes[22,23].

1.2. DIAGNOSIS AND DIAGNOSTIC CRITERIA FOR DIABETES MELLITUS

The body is able to keep glucose level in stable condition. The normal fasting blood sugar varies between 63 and 120 miligrams per deciliter (mg/dL). It would rarely increase up to 144 mg/dL after a diet.

Diagnosis of the diabetes is often prompted by symptoms such as increased thirst and urine level, in severe situations, drowsiness and coma, high level of glycosuria are usually present. Under a concentration of 180 mg/dL, glucose is reabsorbed back into bloodstream by the kidneys and does not found in the urine unless its level in the blood is too high.

Glucose tests that are used to diagnose diabetes mellitus detect the level of blood sugar in a person's bloodstream. Fasting plasma glucose test (FPGT) is designed to measure the level of blood sugar. FPG is usually performed in the morning because that will make it easier for the patient to fast for eight hours. An FPG result below 100 mg/dL is considered normal. Level of glucose results between 100 and 125 mg/dL are accepted prediabetes and glucose level above 125 mg/mL represents diabetes. If there is any doubt about diagnosis, additional tests such as oral glucose tolerance test (OGTT), C-Peptide test, Autoantibody testing and thyroid blood test may be performed. OGTT measures how the body responds to a glucose. A drink contains 75 mg of glucose is given to a patient who has been fasting for 8 hours and after two hours another blood sample is taken. According to the results of glucose tolerance test, the disease can easily be diagnosed.

1.3. INCIDENCE OF DIABETES MELLITUS

Incidence of diabetes increased in the last years and it is estimated that it will keep rising. Obesity and physical inactivity contribute to increasing rate of diabetes mellitus. Diet high in calories, saturated fats and aging of the population are also factors contributing to prevalence of diabetes mellitus.

Epidemiologic studies show that Type I diabetes accounts for five-10 per cent of all cases of diabetes. Incidence rates of Type I diabetes are divided into five groups. 1) very low, < 1/100 000 per year, two) low, 1–4.99/100 000 per year, three) intermediate, 5–9.99/100 000 per year, 4) high, 10–19.99/100 000 per year, and five) very high, 20 or greater /100

000 per year. China, Caracas and Venezuela have a very low prevalence rate (1/100.000), while the greatest rate is observed in Finland and Sardinia (36,5/100.000 and 36,8/100.000)[24].

Type II diabetes is the most common type of diabetes and accounts for 90-95 per cent of all cases of diabetes mostly seen in developed countries. Lastly, about four per cent of all women who are pregnant develop gestational diabetes.

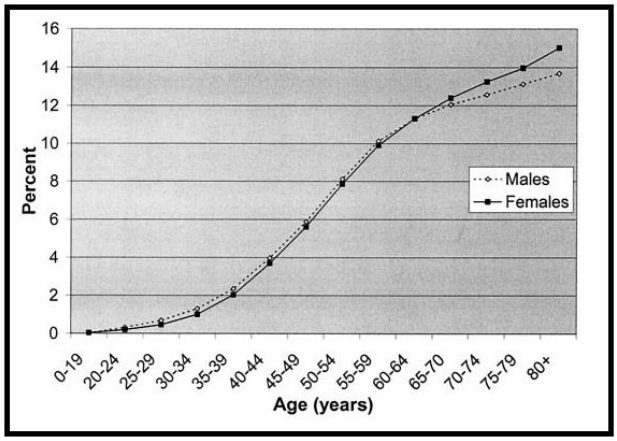

Figure 1.2. Global diabetes prevalence by age and sex for year 2000 [25]

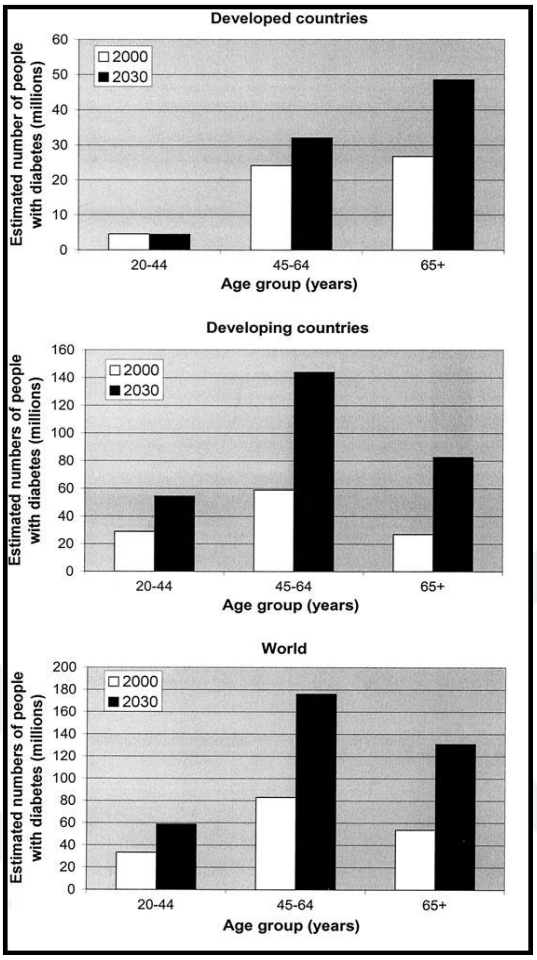

Figure 1.3. Estimated number of adults with diabetes by age-group, year, and countries for thedeveloped and developing categories and for the world [25]

1.4. TYPE I DIABETES, PATHOGENESIS AND INTRINSIC FACTORS

1.4.1. Autoimmune Diabetes Mellitus

Type I diabetes (juvenile diabetes or insulin dependent diabetes mellitus) is an autoimmune disease and defined by hyperglycemia. In type I diabetes the immune system of the host body disrupts the pancreatic beta cells leading to the hyperglycemi. [2]. Destruction speed of pancreatic β-cells can vary from person to person[3]. Rapid destruction of β-cells is mostly seen in children, scarcely it may also be seen in adults[26]. Children and adolescent may present ketoacidosis, which is an acute, major and life-threatening complication of diabetes that mainly seen in patients with type I diabetes. Modest fasting hyperglycemia

may transform into severe hyperglycemia and/or even ketoacidosis in the presence of stresses such as infection. Patients with type I diabetes lose 70-90 per cent of their pancreatic β-cells. Generally remaining cells in adult patients are sufficient to prevent ketoacidosis [2,27]. Patients who have this type of diabetes mellitus become addicted to insulin for survival and are at increased risk for ketoacidosis[4]. Other mammalians such as dogs, cats and other animals can also have diabetes[28].

Immune destruction markers such as islet cell antigen-2 (Insulin-associated antigen-2) (IA-2), autoantibodies to insulin and autoantibodies to glutamic acid decarboxylase (GAD_{65}) are seen in 85-90 per cent of patients with type I diabetes when fasting hyperglycemia is detected [29-34]. Radioligand binding assays show that transcribed and translated GAD65 and IA-2 display elevated sensitivity and specificity for type I diabetes. Susceptibility of insulin autoantibodies depends on age and it decreases as age advances[35].

Table 1.1. Diagnostic sensitivity and specificity of autoantibodies for type 1 diabetes

Autoantigen	**Sensitivity, %**	**Specificity, %**
Insulin	40-70	99
GAD65	70-80	99
IA-2	50-70	99

GAD is the main islet cell autoantigen. Therefore, GAD has been comprehensively studied. In 1990, 64 k-D antigen in the pancreatic β cells of IDDM patients has been identified as glutamic acid decarboxylase by Baekkeskov et al[36]. GAD is expressed in ovaries, thymus, brain, testes, human pancreatic α, β and polypeptide producing cells. It is localized in synaptic-like microvesicles in β-cells. Two different form of GAD has been found, GAD65 and GAD67. These two forms of GAD are nearly 70 per cent homologous according to amino acid sequences. The expression of these two forms of GAD in the pancreatic islet cells is quite different in the species. GAD65 is mainly expressed in rat and human islets. However, GAD67 is expressed in mouse islets [37,38].

HLA (human leukocyte antigen) associations, with the linkage to the DQA and DQB genes, are observedin type I diabetes. HLA region includes a cluster of genes and these are located on chromosome 6. The genes found on chromosome 6 encode some glycoproteins which are found on the cell surface and help the immune system to distinguish the cells. Autoimmune disease occurs when the immune system begins attacking against the tissues of the body. Autoimmune diseases can be associated with the alleles of HLA genes and type I diabetes is one of these disease in which HLA alleles may promote the risk of developing diabetes. Proteins encoded by HLA genes are called major histocompatibility complex (MHC). The inheritance of HLA alleles are able to account for half of the genetic risk to develop type I diabetes[39]. Class II MHC proteins encoded by HLA genes are linked to diabetes. These genes include HLA-DR, HLA-DQ, and HLA-DP. Generally, half of the people inherit copy of DR gene (DR3 and DR4), and only about three per cent of the people have both of alleles. There is a variation in type I diabetes. One allele of DR3 and DR4 is found in about 95 per cent of Caucasians. People having both DR3 and DR4 are prone to develop type I diabetes [40]. However, DR2 has a protective effect [41]. People with specific alleles of DQ gene are also prone to develop type I diabetes. Alleles of DR and DQ gene which have protective effect are usually inherited together.

Transgenic animals have also been used to show the role of GAD in the pathogenesis of type I diabetes. Over expression of GAD in NOD(Non-Obese Diabetic) mice induced the diabetes [42].

First identified antigen for Type I diabetes was insulin. Insulin antibodies can be detected in the early stages of type I diabetes, prior to insulin treatment [30]. It has been shown that the oral intake of insulin postpones disease development in the NOD mouse [43]. Insulin B chain is the main autoantigen which triggers type I diabetes. $CD4^+$ T cell clones specific for insulin B chain detected in NOD mouse accelerate diabetes in young NOD mice [44]. It has been demonstrated that $CD8^+$ T cell clones first recognize insulin B chain amino acids located at the position of15-23 and it causes diabetes in neonatal NOD mouse [45]. Mutations occur in the insulin receptor may also cause hyperglycemia or even symptomatic diabetes [14,15]. People having these mutations may develop acanthosis nigricans which is a skin disorder makes the skin darker and thick. Antibodies for anti-insulin receptor may result in diabetes. They bind to the insulin receptor and reduces the binding of insulin to target tissues [46]. More than 59 per cent of people with diabetes type

I have anti-insulin antibodies. These antibodies can also act as insulin agonist and cause hypoglycemia. Patients with systemic lupus erythematosus also have anti-insulin receptor antibodies [47,48]. Anti-insulin antibodies (IAAs) can be detected by RAI (Radioimmunoassay) or ELISA,both of which are commercially available. However, recommended technique to detect IAAs is RAI [49] due to the sensitivity of RAI is higher than ELISA.

IA-2 autoantigen was identifed in 1994 and it is considered as a major target of immune system of the patients with type I diabetes [33]. It is also shown that IA-2, which is a member of the protein tyrosine phosphatase (PTP) family, is a basic autoantigen in type I diabetes[50]. Bonifacio et al have demonstrated that IA-2 protein is the precursor to the 37 or 40 kD islet tryptic. Autoantibodies against IA-2 are observed in 70 per cent of patients with type I diabetes. The IA-2 autoantigen coming from RIN5AH, which is a rat β-cell line, reacts with serum from IDDM patients fragment [51]. IA-2 is an intrinsic membrane protein in some type of cells including pancreatic cells[52]. It has been revealed that the antigenic targets of autoantibodies against IA-2 are mostly found in the PTP domain [53]. Bingley *et al* have shown that anti-IA-2 autoantibodies has a 58 per cent of sensitivity in IDDM patients [54]. However, in a recent study,the prevalence of those autoantibodies has been indicated as 73 per cent [55]. There are two ways to detect anti-IA-2 autoantibodies: RIA and ELISA assays which are commercially available. The recent ELISA systems used for the detection of anti-IA-2 autoantibodies are as sensitive as RIA[56].

There are evidences affirming to the link between Zinc and diabetes. Hypozincemia is one of the disorder seen in type I diabetes [57,58]. It has been shown that the development of type I diabetes had been inhibited by Zinc supplementation[59]. In 2007, zinc transporter 8 (ZnT8) has been identified as a new marker for type I diabetes[60]. Furthermore, it has been demonstrated that autoantibodies against ZnT8 have been detected in 60-80 per cent of Caucasian patients with IDDM[61]. ZnT8 protein is encoded by *SLC30A8* gene which is located on chromosome 8 in human. Mouse, rat and human ZnT8 are shown 76 per cent amino acid similarity[62]. More than a hundred of genes within the immune cells have been modulated by zinc. It is a necessary element for immune system to function properly[63]. Zinc deficiency triggers symptoms such as thymic atrophy and suppression of T cell response [64]. It was shown that Zinc protects pancreatic β-cells from cytokine-

induced destruction[61]. In addition, the zinc content in β-cells has been increased by the overexpression of ZnT8. Moreover, it protects β-cells from apoptosis [65].

1.4.2. Cellular and Pathogenic Mechanism of Type I Diabetes

Destruction of β-cells of the pancreas by the immune system results in the progression of type I diabetes. Understanding of the pathogenesis of IDDM has been enhanced by studies focused on β-cell autoimmunity using the nonobese diabetic (NOD) mouse [66]. Pathogenic process of the disease is quite complex and studies have shown that autoantigens of β-cells, B lymphocytes, macrophages/dendtiric cells and T lymphocytes are involved in the process. Macrophages and dendritic cells, which are antigen presenting cells, appear earlier, and then $CD4^+$ and $CD8^+$ T cells and B cells appear[67]. It is believed that there are two checkpoints in the pathogenesis of the disease. Checkpoint 1 is the beginning of the disease and tolerance to β-cell antigen is lost in that checkpoint[68] . Non-destructive insulitis captures the islet and becomes destructive in the checkpoint 2. Clinical symptoms of the disease can be detected soon after. There are evidences that β-cells are destroyed by apoptotic mechanisms. Apoptosis of β-cells has been shown in diabetic NOD mice. However, non-apoptotic cell death might be observed [69-71].

1.4.3. Role of Macrophages

It was shown that cells infiltrating the islet are macrophages and dendritic cells. This infiltration occurs in the early stage of insulitis and precedes T lymphocytes and B lymphocytes invasion. Electron microscopy indicated that single cell presenting in the early stage of insulitis is macrophage[72]. In NOD mice, macrophage has been inactivated and then prevention of insulitis and diabetes have been observed. First of all, requirement of macrophages for the improvement of effector T cells that demolish β-cells was displayed. In further study, transplanted NOD islets have not been destroyed by T cells in macrophage-free NOD recipients. Consequently, T cells can not differentiate into cytotoxic T cells that destroy β-cells in a macrophage-free environment [73]. However, these T cells regain their cytotoxic feature if they replace into a macrophage-containing environment. It

is concluded that macrophages have an important role in the improvement and activation of T cells that destroy pancreatic β-cells in NOD mice[252].

Other factors which may conduce to the destruction of β-cells of the pancreas have been investigated. Oxygen-free radicals and cytokines such as IL-1(Interleukin-1), tumor necrosis factor (TNF)-α and IFN-γ(Interferon gamma) have been sough and it has been found that expression of these cytokines has reduced in macrophage-free NOD mice compared to control NOD mice. Cytokines secreted by macrophages are considered to be toxic for β-cells[74,75]. β-cells are quite tender to free radicals such as hydrogen peroxide and superoxide anion. Cytokines produced by macrophages may increase β-cell damage by initiating the production of oxygen-free radicals[76] because free radical scavenging activity of the β-cells of the pancreas are very low.

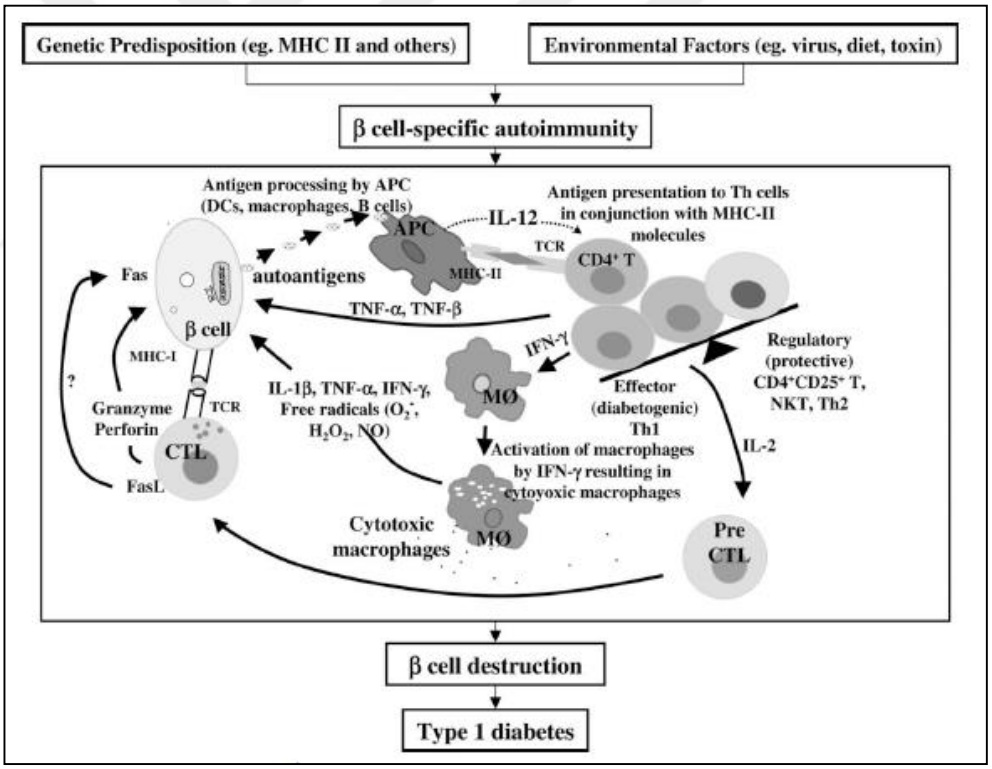

Figure 1.4. Roles and activation of macrophages in type 1 diabetes [267]

1.4.4. Role of B Cells

Studies revealed that B cells also have a critical role in the destruction of pancreatic β-cells. Diabetes has not developed in B cell-deficient NOD mice. Co-receptors consisting of CD81, CD19 and CD21 are expressed by B cells as well as T cells and expression of co-receptors increase the ability of B cells to respond to antigens[253].

1.4.5. Role of T Cells

It is known that T cells have crucial contributions in the destruction of pancreatic β-cells in NOD mouse. In previous studies, it has been shown that insulitis or diabetes has not developed in athymic NOD mouse model [77,78]. NOD mice treated with anti-CD antibodies that binds to CD3 on the surface of T cells inhibited the development of diabetes[78]. $CD4^+$ and $CD8^+$ are needed for the progression of disease[79,80]. There are lots of studies showing the requirement of $CD4^+$ and $CD8^+$ cells for the transfer of diabetes[81,82]. One of the main mechanisms of β-cell destruction is $CD8^+$ T cells-mediated β-cell killing. It was revealed that $CD8^+$ T cells can eradicate β-cells by activating MHC class I served on pancreatic β-cells. It was indicated that deficiency in MHC class I is enough to arrest development of diabetes and prevent destruction of β-cells of the pancreas in NOD mouse[83].

The importance of $CD4^+$ and $CD8^+$ cells in type I diabetes were also studied. It has been shown that although $CD4^+$ cells attached to the β-cells of the pancreas, they did not destroy β-cells. However, $CD8^+$ cells had cytotoxic effects on β-cells. $CD8^+$ cells exhibited some protrusions, which looked like pseudopod, into β-cells but not into other cell types of the pancreas such as α and γ cells. This situation leads to selective destruction of β-cells[84]. It is concluded that the interaction of β-cells with $CD4^+$ and $CD8^+$ cells are quite different during the T-cell-mediated destruction of β-cells. Cytokines such as IL-2 and INF-γ can be secreted by $CD4^+$ cells and these cytokines activate $CD8^+$ cells. $CD8^+$ cells also secrete cytokines which in turn may upregulate Fas within the islets. Actually, Fas in the islets can be upregulated by $CD4^+$ and $CD8^+$ cells, by secreting cytokines such as IL-2 and INF-γ. If Fas on the islet is upregulated, apoptosis may be induced by FasL-expressing $CD8^+$ T cells and $CD4^+$ Th1 cells.

As it was discussed above, cytokines have crucial roles in the pathogenesis of β-cells in type 1 diabetes. Th1 cytokines such as (IL-2, IFN-γ) play a role in the development of diabetes. Nevertheless, Th2 and Th3 cytokines such as IL-4, IL-10 and TGF-β take in charge in the prevention of the disease. Studies revealed that development of the disease has been prevented in anti-IFN-γ-treated NOD mice. In contrast, overexpression of IFN-γ in the β-cells of a non-diseased mouse causes the development of diabetes type I. In addition, IL-4 or IL-10 treatment in NOD mouse and overexpression of IL-4 in the β-cells prevent diabetes [85].

T cell mediated-β-cell destruction is done by Fas-FasL interaction as well as granzyme and perforin pathways. It has been shown that diabetes or insulitis did not develop in Fas-deficient NOD mice and studies performed in 1997 and 2000 suggested that Fas-mediated apoptosis may be the main mechanism of β-cell destruction [86,87].

1.4.6. Role of Fas/Fasl Interaction

The Fas receptor (FasR), also known as CD95, is a receptor found on the cell surface and it mediates apoptosis. FasR is located on chromosome 19 in mice. High level of Fas is seen in mouse thymus, heart, liver, and ovary and activated lymphocyte[88]. Cytokines such as IFNγ, IL-1 and TNFα upregulate the expression of Fas in the number of cell types. FasL, which is expressed only on activated T cells, is a membrane protein and it is also known as CD95L. Programmed cell death (apoptosis) activated by FasL has tasks such as removal of lymphocytes after an immune response. Destruction of β-cells by Fas-FasL interaction occurs under two circumstances. Firstly, β-cells express Fas and they coexpress FasL or β-cells are able to interact with FasL expressed on the other cells present in the islet of langerhans (e.g., T cells infiltrating islet or α-cells). To show the role of Fas in development of diabetes, scientists have used NOD*lpr* mice which are Fas-deficient. Studies performed with NOD*lpr* mice showed that Fas plays major roles in the improvement of T1D.[89,86]. Even though diabetic NOD splenocytes are transferred to NOD*lpr* mice, insulitis or diabetes does not develop[90]. It is also shown that NOD-gld/gld mice,which are Fas-deficient, did not develop insulitis or diabetes[87]. According to these results, Fas/FasL interaction is essential for the development of T1D. Secondly, the overexpression of FasL in β-cells of transgenic NOD mice accelerated the disease[89].

These results suggest that Fas-FasL-mediated cytotoxicity is a major issue in the destruction of β-cells. However, islets grafts,which are Fas-deficient, from NOD*lpr* mice was not able to survive in diabetic NOD mice[91]. NOD diabetes induced by cyclophosphamide has not been blocked by anti-FasL antibody[92]. It was also shown that < five per cent of β-cells expressed Fas in prediabetic NOD mice[93]. These evidences indicate that Fas plays a minor role in destruction of β-cells of the pancreas. Consequently, the main role of Fas/FasL-mediated cytotoxicity is still arguable.

1.5. OXIDATIVE STRESS AND TYPE I DIABETES

Oxidative stress has a critical role in the development of diabetic complications[94]. Formation of highly reactive molecules including Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is defined as oxidative stress[95,96]. Free radicals such as superoxide (•O2-), hydroxyl (•OH), peroxyl (•RO2) and hydroperoxyl (•HRO2-) are included in ROS. Whereas, hydrogen peroxide (H2O2), nitric oxide (NO), peroxynitrite (PON) and hydrochlorous acid (HOCl) are included in RNS[95,97,98]. The main source of free radicals is glucose oxidation. The superoxide anion radicals produced by glucose oxidation undergo dismutation. Thus, hydrogen peroxide is formed. Hydrogen peroxide has to be degraded by catalase or glutathione peroxidase. If hydrogen peroxide is not degraded, highly reactive hyrdoxyl radicals are performed[99,100].

As it has been mentioned before, inflammatory cytokines and ROS are secreted by activated macrophages. Moreover, ROS is also produced as a byproduct of oxygen metabolism and have several important roles in cell signaling and homeostasis. The sources of ROS in cell vary. Nonenzymatic glycosylation reaction, the electron transport chain which takes place in mitochondria and membrane-bound NADPH are some of the sources of ROS[101-104]. It was demostrated that ROS is also generated in different tissues under diabetic conditions[105,106]. NADPH, which is a source of ROS, is activated by several stimuli such as insulin and angiotensin II. Electron transport chain is also induced under diabetic conditions. ROS is generated by several species under normal physiological conditions and scavenged by antioxidant system encompasses glutathione peroxidase, catalase and superoxide dismutase (SOD). This equilibrium is eventually lost in diabetic people because ROS production is enhanced and the antioxidant protective

mechanism of the organism is decreased. Oxidative stress causes cellular dysfunctions which have important roles in the development of diabetic symptoms and β-cell dysfunction[107,97,108,109]. β-cells are unprotected from oxidative stress because the expression level of antioxidant enzymes such as superoxide dismutase 1 and 2 (SOD1-2) , catalase (CAT) and glutathione peroxidase (GPx) are very low.[110-112]. According to Modak et al., low DNA repair capacity against oxidative stress is shown in islets[113].

It has been shown that hyperglycemia triggers peroxidation of low density lipoprotein (LDL) resulting in the production of free radicals[114,115]. The interaction of glucose with proteins is another source of free radicals in diabetes and this interaction leads to firstly the formation of an Amadori product and then advanced glycation end products (AGEs)[116,117]. AGEs deactivate enzymes by using their receptors (RAGEs), change their structure and finally cause some abnormalities in enzyme functions[118]. It has been reported that AGEs also induce the formation of free radicals[105,119]. AGEs activate stress-related pathways such as NF-ĸB (nuclear factor kappa-light-chain-enhancer of activated B cells). This activation enhances upregulation of several genes controlled by NF-ĸB[120].The production of nitric oxide is enhanced by NF-ĸB. It is believed that nitric oxide plays a role in β-cell damage.

ROS directly damage DNA, lipids and proteins. Thus, it is believed that ROS have major roles in the pathogenesis of late complications of diabetes. Moreover, ROS can also act as signaling molecule activating several cellular stress-sensitive pathways which cause cellular damage and are responsible for decreased insulin secretion and insulin resistance[121]. It has been indicated that oxidative stress caused by hyperglycemia activate biological pathways including the stress-activated signaling pathways such as NF-ĸB, NH2-terminal Jun kinases/stress activated protein kinases (JNK/SAPK) p38 mitogen-activated protein (MAP) kinase, hexosamine.

1.5.1. Role of NF-κB Pathway

The main reason for the β-cell death is apoptosis which is a highly organized process, arranged by extracellular signals, the level of intracellular ATP and pro-antiapoptotic gene expressions. β-cell death during insulitis, which is an inflammation of the islets of Langerhans of the pancreas, is caused by the interaction between activated macrophages

and T cells. Cytokines released by these cells also play a role in the inflammation.[122]. Cytokines trigger stress response genes which can be protective or harmful. It was shown that *in vitro* exposure of β-cells to IL1-β + interferon (IFN)-γ results in physiological changes which are similar to those seen in pre-diabetic patients[123]. NF-ĸB is a protein complex and controls the DNA transcription. It is found in animal cells and included in cellular response against stimuli such as ultraviolet irradiation, cytokines and free radicals. Abnormal regulation of this pathway is seen in autoimmune diseases. In human and rodent β-cells, NF-ĸB is activated by IL1-β[122]. Activation of NF-ĸB is prevented by inhibitory ĸB (IĸB) "super-repressor", and pancreatic β-cells are protected against cytokine-induced apoptosis. [124,125]. NF-ĸB downregulates some of transcription factors(PDX-1 and Isl-1) which are responsible for action and differentiation of pancreatic β-cells. It was shown that an NF-ĸB inhibitor is transgenically expressed under the control of pdx-1 promoter. Moreover, imperfect GLUT-2 (Glucose transporter 2) expression and insulin release are seen. Thus, it can be concluded that basal level of NF-ĸB is necessary for normal insulin secretion [126].NF-ĸB regulates the expression of genes resulting indiabetic complications(e.g. vascular endothelial growth factor [VEGF] and the receptor for advanced glycation endproducts (RAGE)[120].Cells normally respond to oxidative stress, which is caused by hyperglycemia, by modulatig the expression of antioxidant enzymes such as superoxide dismutase, glutathione peroxidase and catalase. Exposure of cells to H_2O_2 may regulate NFĸB. [127,128]. Hyperglycemia stimulates intracellular ROS production and then activation of NFĸB. It was also shown that H_2O_2-induced NFĸB activation depends on cell type. Thus, H_2O_2 is not a general mediator for NFĸB activation[129-131].

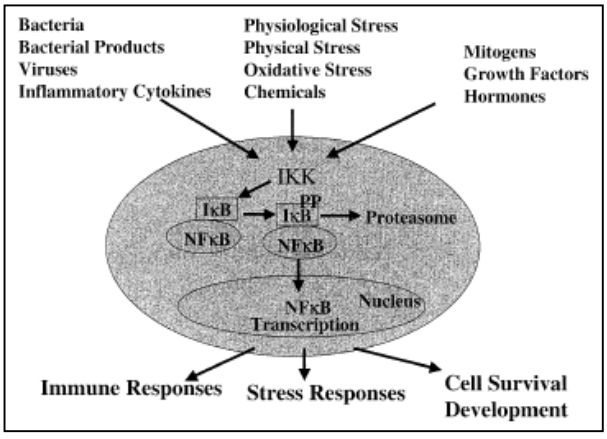

Figure 1.5. The activation of NFĸB pathway and its responses [254]

1.5.2. Role of (Jnk/Sapk) Pathway

c-Jun N-terminal kinases (JNK) , also known as Stress Activated Protein Kinase (SAPK), bind and phosphorylate c-Jun. They belong to MAPK family. They are activated by stress stimuli such as ultraviolet irradiation, ROS, heat shock and cytokines. JNK activation is involved in insulin gene expression and β-cells are protected from oxidative stress if JNK pathway is suppressed[132]. Studies on β-cells demonstrated that IL-1β was found to mediate the JNK activation.[133-135]. Inhibition of JNK pathyway showed that the protection against β-cell apoptosis induced by IL-1β in insulin-producing cells[136-138]. TNF-α and IFN-γ can activate the cytotoxic effect of IL-1β on β-cells and these two cytokines increase IL-1β-induced signaling by the JNK pathway[139].

It was shown that JNK2 has major roles in type I diabetes resulted from autoimmune destruction of pancreatic β-cells[140]. Studies performed on NOD mice revealed that deterioration of JNK2 led to decreased insulitis and reduced the progression of the disease. Isolated $CD4^+$ T cells from JNK2-deficient NOD mice generated fewer IFN-γ. Therefore, it can be concluded that JNK pathyway plays an important role in destruction of β-cells of the pancreas in type I diabetes.

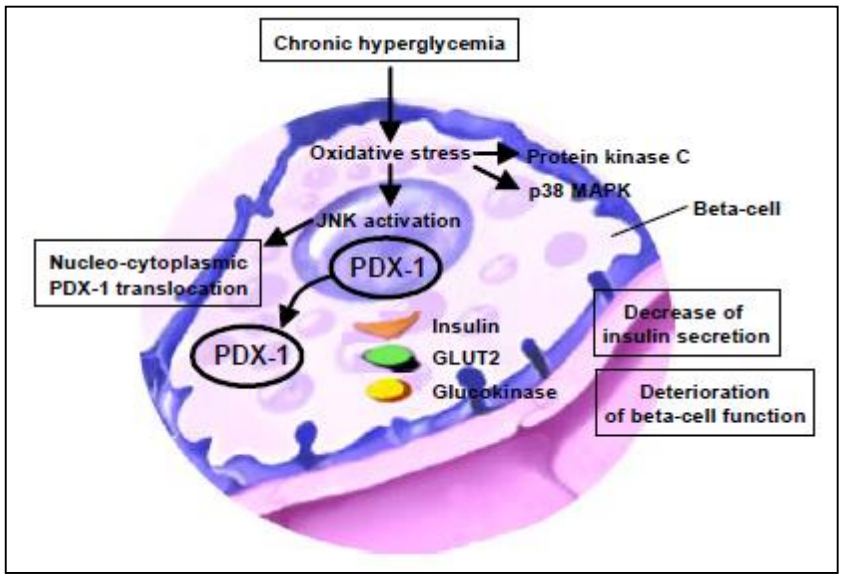

Figure 1.6. The JNK pathway and beta cell dysfunction [255]

1.5.3. Role oF p38 Map Kinase (MAPK) Pathway

p38MAP kinases are serine/threonine-specific protein kinases and involved in cellular responses to several stimuli such as osmotic stress, heat shock and secretion of proinflammatory cytokines. Differentiation, cell-survival, gene expression and apoptosis are regulated by those kinases. p38 MAPK family has four members: p38*α*, p38*β*, p38*γ*, and p38*δ*. p38α has been identified as a 38kDa polypeptide and it is activated by cell stress and cytokines[141]. In diabetes, it was shown that hyperglycemia triggers p38 MAPK activation[142]. In a study performed in rat aortic smooth muscle cells, hyperglycemia results in an increase in p38 MAPK activation. In nerve tissue of patients having type one and type two diebetes, increased p38 MAPK and JNK/SAPK activitiy have been observed[143]. p38 MAPK is an important protein that regulates the production of inflammatory cytokine and adjust the function of T cells[144,145]. It has been demonstrated that p38 MAPK pathway is active in T cells of NOD mice and adjusts the early stage of T cell development[146-148]. p38 MAPK modulates differentiation and proliferation of T cells and regulates T cell function encompassing $CD8^+$ cytotoxic T cells, $CD4^+$ helper cells and the production of IL-4[146,145,149,148].It was shown that treatment of p38 MAPK inhibitor (FR167653) decreased insulitis and β-cell destruction in NOD mice[150].

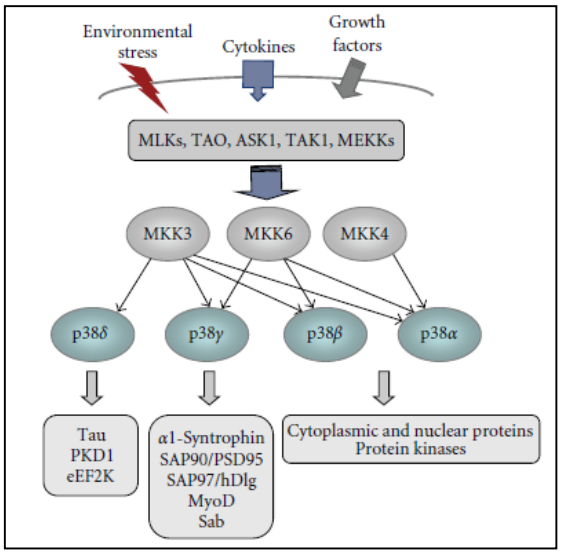

Figure 1.7. The p38MAPK pathway and its members [256]

Heat shock proteins (HSPs) are crucial proteins which funtion within T cells in stress-induced conditions such as autoimmune diseases and infection[151]. HSP60 has been shown to be a diabetes-associated autoantigen and functions in T cell responses in which Th1-dependent inflammatory situations such as insulitis[152-155]. It was demonstrated that there is a correlation in between the expression of p38 MAPK and HSP60. Blocking p38 MAPK has decreased the expression of HSP60 in several cell lines[156,157].Medicherla *et al.* have shown that SD-169, which is a selective inhibitor for p38α, can protect the development of diabetes in NOD mice. Treatment with SD-169 has positive impacts including reduced T cell infiltration, recovery in glucose tolerance, increased insulin level and decreased HSP60 expression as well as p38 MAPK [158].It can be concluded that p38 MAPK and HSP60 are functionally linked.

1.5.4. Role of Hexosamine Pathway

Destruction of β-cells of the pancreas results in insulin resistance. It can be tolerated by normal cells. They increase insulin secretion and the mass of β-cells. In case of hyperglycemia, the function of β-cells deteriorates [159] and can cause insulin resistance in humans[160] and in animal models[161]. Excessive uptake of glucose into many cell types causes hexosamine biosynthetic pathway activation[162,163].

In the hexosamine pathway, glutamine:fructose-6-phosphate amidotransferase (GFAT) converts fructose-6-phosphate to *N*-acetylglucosamine-6-phosphate. In the subsequent step, *N*-Acetylglucosamine-6-phosphate is converted to *N*-acetylglucosamine-1,6-phosphate and to UDP-GlcNAc, respectively. UDP-Glc-NAc serves as a substrate for *O*-linked glycosylation. The *O*-linked glycosylation is an important event and reversed by *O*-GlcNAc _-*N* acetylglucosaminidase[159]. There are studies showing the correlation between the hexosamine pathway and *O*-GlcNAc modification[164,165]. There are reports showing that *O*-Glc-NAc modifies several proteins encompassing some of the transcriptional factors and gene transcription can be activated by this modification[166,165,167,168]. It was reported that the hexosamine pathway has a crucial role in insulin resistance[169,170]. The elevated expression of GFAT in muscle, fat and liver causes insulin resistance[171,170]. It was shown that *O*-GlcNAc modification of key transcription factors makes contribution to insulin resistance[172] andthe overexpression

of *O*-GlcNAc transferase is seen in β-cells of the pancreas[173,174]. Furthermore, streptozotocin, which is a diabetogenic reagent, increases the level of *O*-GlcNAc in islets. In the lights of these observations, it can be concluded that hexosamine pathway plays major roles in the action and the destruction of β-cells.

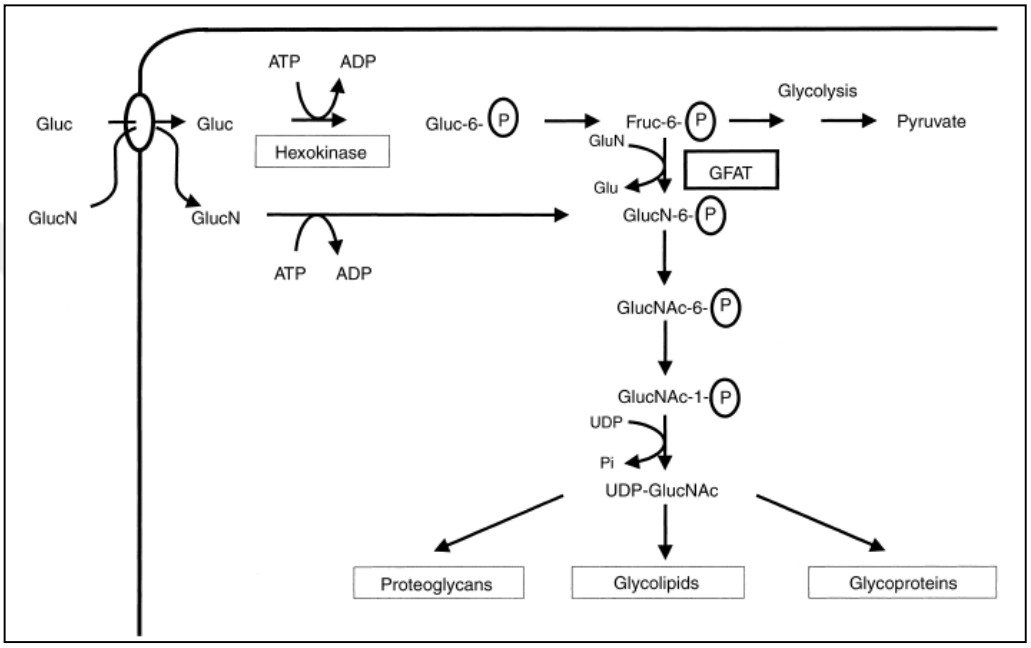

Figure 1.8. The hexosamine biosynthetic pathway [257]

In brief; NF-ĸB, hexosamine, p38 MAPK, and JNK/SAPK pathways are stress-sensitive pathways which can be activated by ROS and hyperglycemia *in vivo* and *in vitro*. The late complications of diabetes are associated with the activation of these pathways. There are several reports indicating the activation of these pathways to β-cell dysfunction and to insulin resistance.

1.6. BORON

The fifth element in the periodic table, boron is an important element found in the nature. It is a semiconductive semimetal and has an atomic number of five [175]. Boron is symbolized with “B”. It has metal and nonmetal physical properties. B is found in the

nature as ^{11}B and ^{10}B isotopes[175]. B can not be found in cation form in the nature and is quite reactive with oxygen. Boron always generates B-O bonds in natural conditions. It is tend to create complex structures with organic molecules which have hydroxyl groups. It can interact with crucial biological substances such as pyridoxine, the pyridine nucleotides and riboflavin[176]. Borates, boric acid (H_3BO_3 or $B(OH)_3$) and borosilicate minerals are the natural forms of Boron [177]. According to literature, boron is found in the form of boric acid with a small quantity of borate at natural pH in biological fluids. Boric acid can dissolve as shown below [178];

$$B(OH)_3 + H_2O \Leftrightarrow B(OH)_4^- + H^+ \quad (1.6)$$

Boron serves as an important element for plants development and contributes to cell wall structure as well as function[179]. It has a role in cell wall cross-linkingand binds to fibrous component of the cell wall[180]. Furthermore, Boron plays important roles in carbohydrate metabolism, nucleic acid synthesis, especially in elevated synthesis of RNA and hormone function[180,181]. In addition, it was shown that higher organisms such as mouse, frog, buffalo and zebrafish need boron as well to maintain their health[182,183]. Till 2004, boron transport was believed to be through passive diffusion. However; in 2004, Park *et al.* have discovered that boron transport occurs through Electrogenic Na^+-Coupled Borate Transporter (NaBC1)[184].

The relationship between boron and human health have been revealed with studies on bone growth. It was shown that boron deficiency diminished the growth of pig and rat bone [185]. In addition, alveolar bone loss and periodontal inflammation has been decreased by systemic boron supplementation[186]. In osteoblasts, bone-related protein expressions and mineralization level have been increased by boric acid which is a boron-derivative [187].Boron deficiency results in urinary calcium excreation in animals as well as humans [176]. In addition, boron deprivation aggravates the signs of vitamin D_3 deficiency such as impaired bone formation and growth [188].

There are studies showing the relationship between boron and prostate cancer. *In vitro*, proliferation of prostate cancer lines DU-145, PC-3 and LNCaP has been decreased by

boric acid treatment [189]. Other than prostate cancer, proliferation of lung, breasts and cervical cancers has been inhibited by boron treatment *in vivo* [190-192]. Although mechanisms is yet to be determined, it can be stated that boron has anti-tumourigenic effect on many different cancer types.

Immunity and oxidative stress regulatory effects of boron have also been discussed in the literature. Accelerating boron supplementation in diet has diminished the circulation of natural killer cells [193]. In addition, the treatment of low dose boron has increased antioxidant enzyme activity [194]. Boron also lowers the amount of IL-6, nitric oxide (NO), and IL-1β which induce apoptosis of pancreatic β-cells.

There are studies showing the effect of boron on magnesium, vitamin D and calcium metabolism. It was shown that the addition of boron attenuates the level of impairments caused by insufficient magnesium intake [195]. Boron supplementation triggered growth and augmented the level of magnesium and calcium level in the plasma [195]. Nielsen *et al.* indicated that insufficient boron in diet aggregates symptoms of magnesium deficiency in magnesium-deficient rats [196]. In 1981, Hunt and Nielsen represented the relationship between boron and vitamin D in chicks. Boron supplementation up to three mg/kg increased growth by 38 per cent, augmented plasma calcium concentrations and reduced alkaline phosphatase level in chicks which are vitamin D-deficient and consumed basal diets containing three mg/kg [197]. It was also shown that boron supplementation successfully reduced the side effects caused by vitamin D-deficiency including elevated triglyceride, cholesterol and plasma glucose levels [198].

Boron also has effect on wound healing processes. Skindeep boron treatment and thermal water boron are associated with wound healing properties [199]. Treatment with three per cent boric acids, which is a boron-derivative, has assisted healing process and reduced duration of stay in the hospital for treatment [200,201]. The complete mechanism of boron on wound healing process is not known yet. Some molecular changes in cells after boron application have been revealed by limited number of studies. In 1998, Benderdour and colleagues represented that the application of boric acid has increased the secretion of Extra Cellular Matrix (ECM) proteins such as collagen and proteoglycans and TNF-α [202]. Moreover, it has also been shown that four boron counterparts (2,2-dimethylhexyl-l,3-propanediol-aminopropylboronate,triethanolamine borate, 1,2-propanediol-aminopropyl-boronat and N-diethyl-phosphoramidate-propylboronique acid) enhanced the

level of ECM proteins more than boric acid did but they were more toxic than boric acid[203]. Correlation between VEGF (vascular endothelial growth factor) and boron treatment has been shown by Dzondo-Gadet *et al*. in 2002. Boron application marked up VEGF expression in both RNA and protein level [204]. It has been shown that BA and NaB are influential against *E. coli, A. niger* and *S. aureus* and MIC levels for NaB and BA are different [258, 259]. Although BA and its salts have been used as fungicide and bactericide, molecular mechanism of antimicrobial effects are still completely unknown. This feature of boron is not an enough reason to be used in a wound care product. Moreover, anti-inflammatory properties of boron derivatives were investigated. It has been shown that NaB and BA treatment reduced the production of NO and suppress COX-2 and iNOS which are inflammatory mediator genes. In addition, another boron compound, calcium fructoborate, reduced protein level of IL-6 and IL-1β [260]. Boron derivatives have also an effect upon growth factors of keratinocytes and human fibroblast. It has been shown that granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage-colony stimulating factor (GM-CSF) are upragulated by boron derivatives treatment. G-CSF enhances wound healing process by increasing angiogenesis, while GM-CSF promotes tissue formation, endothelial cell migration, proliferation and re-epithelization. These findings suggest that boron derivatives might be used in wound healing process of ischemic diseases such as diabetes [228].

In 1998, it was reported that boron influenced upon at least 26 different enzymes found in plant, animal, chemical and cultured reaction systems. Boron has roles in the regulation of enzymes involved in the immune system, insulin release and energy substrate metabolism. Glycolytic enzyme activity is blocked by boron. It has been shown that boron regulates insulin release by changing NADPH metabolism. Alterations in the metabolism of NADPH result in alterations seen in the dynamics of the cell membrane, resulting in insulin secretion [205]. The activity of two essential enzymes in Pentose Phosphate Pathway (PPP) including glucose-6-phosphate dehydrogenase and GPD is inhibited by boron [205]. The inhibitory effect of boron on enzymes in PPP may modify the level of NADPH. In 1998, Hunt has shown that boron may also attenuate oxidative damage level by reducing NADPH production and λ-glutamyl transpeptidase activity[205]. It is possible that this effect could probably augment the quantity of glutathione (GSH) in the cells. Protective effect of GSH from free oxygen radicals is already known [206].

As it has been discussed above, boron has crucial roles for the maintenance and homeostasis for plants and animals. Unique properties of boron can be used to cure diseases related to plants and animals. However, further investigations are needed.

In conclusion, boron is so important for plant and human metabolism. As discussed above, boron has effects on calcium metabolism as well as antioxidant enzyme activity and cell viability which are the main problems in type I diabetes. The effect of boron on type I diabetes has not been studied so far. However, ample evidences obtained from experimental studies suggest that boron and its derivatives may have positive effects on type I diabetes. Therefore, boron derivatives need to be investigated for type I diabetes *in vitro*.

1.7. ADVICES FOR PATIENTS WITH DIABETES

Before medical care of diabetes, factual assessments should be done to classify diabetes. The things to do in the first place are listed below:

- Diabetic complications of the patients should be determined.
- Former treatments and glycemic control in people suffering from diabetes should be checked out
- Proper laboratory tests for patient's medical care should be done

When planning for diabetic cure, some conditions should be taken into consideration. For instance; current signs of diabetes, age of patient, work, school or life conditions of the patient, eating habits, cultural and social conditions.

It is recommended that patients with type I diabetes should use 3 or 4 doses of basal insulin injections per day. For obese or overweight individuals having diabetes, weight loss is strongly recommended due to the decrease in insulin resistance. Changes in certain behaviors and physical activities are also important parts of weight loss[207].

Physical activity should also been changed by people having diabetes. It is advised that diabetic people should perform 150 mins/week of physical activity. It has been shown that

regular exercise helps to evolve blood glucose control, decrease the risk factors of cardiovascular disease and provide loss in weight[208].

1.8. THE AIM OF THE STUDY

Wide range of arguments achieved from experimental studies evoke that boron and its derivatives may have important roles on cell viability and antioxidant enzyme activity. Thus, we hypothesize that boron derivatives may be useful to treat or reduce the symptoms of type I diabetes. Boron compounds may have a role in the survival and the maintenance of β-cells of the pancreas which are main targets in type I diabetes. Previous studies showed that antioxidant enzyme activities of the patients with type I diabetes is quite low. Antioxidant properties of boron may be used to increase antioxidant enzyme activity and the cell viability which are the main problem in type I diabetes.

The current study was performed to; evaluate the effect of boron compounds on cell viability of β-cells, insulin secretion and ROS production, cell surface antigens, apoptotic markers, antioxidant enzyme activities, pathways leading to apoptosis and evaluate changes in cytokines profiles.

2. MATERIALS AND METHODS

2.1. *IN VITRO* STUDIES

2.1.1. Preparation of Boron Solutions

Sodium Pentaborate (NaB), supplied by National Boron Research Institute-BOREN (Ankara, Turkey), and boric acid (BA) (#10043-35-3, Bio Basic Inc., Canada) were dissolved in Dulbecco's modified Eagle's medium (DMEM) (#41966-029, Invitrogen, UK) at a concentration of 10mg/ml. Boron solutions were sterilized by using 0.22 µm filter (#99722, TPP, Switzerland) and subsequently diluted in DMEM for further *in vitro* experiments.

2.1.2. Cell Viability Assay

2.1.2.1. Normoxia

Pancreatic Beta-TC-6 cell line (β-TC-6, ATCC-CRL 11506) was purchased from ATCC (Rockville, MD). Frozen cells were thawed in the 37 °C water bath. The cells were centrifuged at 500 g for five minutes. Supernatant was removed and cells were suspended in DMEM containing 15 per cent fetal bovine serum (FBS) (#10500-064, Invitrogen, UK), and one per cent of penicillin, streptomycin, and amphotericin B (PSA) (#15240-062, Invitrogen, UK). Resuspended cells were cultured in a humidified chamber at 37 °C and five per cent CO_2. Cells were passaged when reached enough confluency.

The effects of NaB and BA on β-TC-6 cells were evaluated using 3-(4,5-dimethyl-thiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium (MTS) assay (#G3582, CellTiter96 AqueousOne Solution; Promega, Southampton, UK) according to the manufacturer's instructions. Different concentrations ranging from 625µg/ml to 600 ng/ml (625µg/ml, 312.5µg/ml, 156.25µg/ml, 78.1µg/ml, 39.06µg/ml, 19.5µg/ml, 9.76µg/ml, 4.88µg/ml, 2.44µg/ml, 1.22µg/ml and 600ng/ml) of BA and NaB were

prepared in DMEM supplemented with 10 per cent FBS and one per cent PSA. Cells were seeded onto 96-well plates (#CLS6509, Corning Plasticware, Corning, NY) at a concentration of 8×10^4 cells/well and cultured for two days in a humidified chamber at 37 °C and five per cent CO_2. After two days incubation period, medium was changed with fresh medium containing aforementioned concentrations of BA and NaB. 20 per cent of dimethyl sulfoxide (DMSO, #D4540, Sigma-Aldrich, USA) was used as positive control. Cell viability for 24, 48 and 72h was measured by adding 10 per cent MTS reagent containing complete growth medium into each wells and cells were incubated at 37 °C for one–three hours in a humidified incubator and five per cent CO_2 atmosphere. Absorbance was qualified at 490nm by using an ELISA plate reader (Biotek, Winooski, VT) [209].

2.1.2.2. Hypoxia

The effect of optimum NaB and BA concentrations on β-TC-6 cells were tested in hypoxic conditions. Firstly, pancreatic β-TC-6 cells were seeded onto 96-well plate at a density of 8×10^4 cells/well and cultured for two days in a humidified chamber at 37 °C, five per cent CO_2 and 21 per cent O_2 (normoxic conditions). After two days of incubation in normoxic conditions, medium in each well was replaced with fresh growth medium and cells were incubated in hypoxic conditions (37 °C in a humidified, five per cent CO_2 and one per cent O_2 atmosphere) for two days. After two days, medium was changed with fresh medium containing 19,5 µg/ml of NaB and BA. As positive control, 20 per cent DMSO was used. Cells were incubated in hypoxic conditions for additional two hours. Cell viability for 48h was measured by adding 10 per cent MTS reagent containing complete growth medium into each well. Absorbance was measured at 490nm by using an ELISA plate reader.

2.1.3. Quantitative Real-time PCR (RT-PCR) Assay

Primers for the target genes were desinged by using Primer-BLAST online software of the National Center for Biotechnology (NCBI, Bethesda, MD) and synthesized by Macrogen (Seoul, Korea). As a housekeeping gene, β-actin was used for the normalization of results. The sequences for primers, RT-PCR reagents and conditions were given in Table 2.1, Table 2.2 and Table 2.3, respectively. Total RNAs from boron treated and untreated cells

were isolated by using High Pure RNA Isolation Kit (#11828665001, Roche, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized by using High Fidelity cDNA synthesis kit (#05081955001, Roche, USA) according to company's advices. Reverse transcription polymerase chain reaction (RT-PCR) with SYBR Green method was used to determine mRNA levels of the target genes. Primers (10pmol for each), cDNA (800ng), dH_2O and Maxima™ SYBR Green qPCR Master Mix (2×) (#K0221, Fermentas, USA) were mixed in a final volume of 20µl. β-actin was used for normalization of data. All RT-PCR experiments were conducted using CFX96 RT-PCR system (Bio-Rad, Hercules, CA).

Table 2.1. Primers used in RT-PCR assays

Gene	**Species**	**Sequence**	**Product length**
Caspase 3	Mouse	F 5' GGGAGCAAGTCAGTGGACTC 3' R 5' CCGTACCAGAGCGAGATGAC 3'	136bp
P53	Mouse	F 5' CAGTGGGAACCTTCTGGGAC 3' R 5' CTTCTGTACGGCGGTCTCTC 3'	77bp
Akt	Mouse	F 5'GGGACCTGAAGCTGGAGAAC 3' R 5' CCTGGTTGTAGAAGGGCAGG 3'	240bp
TGF-β1	Mouse	F 5' AGGGCTACCATGCCAACTTC 3' R 5' CCACGTAGTAGACGATGGGC 3'	168bp
IL-4	Mouse	F 5' CCATATCCACGGATGCGACA 3' R 5' AAGCACCTTGGAAGCCCTAC 3'	166bp
Bcl-2	Mouse	F 5' CCACCTGTGGTCCATCTGAC 3' R 5' CAATCCTCCCCCAGTTCACC 3'	175bp
Insulin	Mouse	F 5' TGGCTTCTTCTACACACCCAAG 3' R 5' ACAATGCCACGCTTCTGCC 3'	132bp
PPI	Mouse	F 5' GGGGAGCGTGGCTTCTTCTA 3' R 5' GGGGACAGAATTCAGTGGCA 3'	86bp
Glucokinase	Mouse	F 5' TAGCGGGGGTCATAAATCGC 3' R 5' TTGTACACGGAGCCATCCAC 3'	85bp

Pdx-1	Mouse	F 5’ CCTTTCCCGAATGGAACCGA 3’ R 5’ GGGCCGGGAGATGTATTTGT 3’	228bp
SOD-1	Mouse	F 5’ AAG CGG TGA ACC AGT TGT GT 3’ R 5’ CTG ATG GAC GTG GAA CCC AT 3’	76bp
SOD-2	Mouse	F 5’ GAA CAA TCT CAA CGC CAC CG 3’ R 5’ ACC TTG CTC CTT ATT GAA GCC A 3’	295bp
Glut2	Mouse	F 5’ TGA GTT CCT TCC AGT TCG GC 3’ R 5’ AGC TTT CCG GTC ATC CAG TG 3’	110bp
β-actin	Mouse	F 5’ GACAGGATGCAGAAGGAGATTACT 3’ R 5’ TGATCCACATCTGCTGGAAGGT 3’	141bp

Table 2.2. RT-PCR reagents

Reagents	**Volume**
Maxima™ SYBR Green qPCR Master Mix	10 µl
Primer Forward (10pmol)	1 µl
Primer Reverse (10pmol)	1 µl
Distilled water	5 µl
Template (200ng/ml)	5 µl

Table 2.3. Real Time PCR conditions

Cycle	**Repeats**	**Step**	**Dwell time**	**Set point**
Initial Denaturation	1	1	3 min	93 °C
Denaturation	36	1	30 sec	93 °C
Annealing		2	40 sec	58 °C
Extension		3	45 sec	72° C
Final extension	1	1	10 min	72 °C
Melt curve	110	1	12 sec	-0.5 °C/cycle
Hold	1	1	-	4°C

2.1.4. Annexin V Apoptosis Detection Assay

Annexin V apoptosis detection assay is used to detect if cells undergo apoptosis. Enlegland et al. clarified the mechanism of action of Annexin [210]. Briefly, pancreatic β-TC-6 cells were treated with 19,5 µg/ml of BA and NaB to investigate whether the boron derivatives cause programmed cell death in hypoxic conditions. Apoptosis has been measured in cells treated with BA and NaB containing medium or normal growth medium by Annexin V (#APT750, ApopNexin^{TM} FITC Apoptosis Detection Kit, Millipore, Temecula, CA, USA) labeling followed by flow cytometry analysis according to the manufacturer's instructions. Apoptosis was incuded by incubation of cells in hypoxic conditions for 24h and 48h. The cells were collected and washed by PBS. 10^6 cells were taken into a chilled culture tube and spinned down at 400 x g for five min. Supernatant was removed and the cells were resuspended in five ml ice-cold PBS and spinned down. Supernatant was removed once more and the cells resuspended in ice-cold 1X Binding Buffer at a concentration of 10^6 cells/ml. 200µl cell suspension was taken and 3µl of annexin conjugated ApopNexin^{TM} FITC was added. Afterwards, two µl 100x Propidium iodide (PI) was added to ApopNexin^{TM} FITC labeled cells. The mixture was incubated for 15 min at room temperature in the dark. Apoptotic cells were evaluated by using Flow Cytometry (FACS Calibur, BD, USA).

2.1.5. SOD (Superoxide Dismutase) Enzyme Activity

The activity of SOD enzyme, which has low expression in type I diabetes, was determined in the current study. The mechanism of action and roles of SOD enzyme were given in different studies[211,212,213,214,215,216]. Shortly, pancreatic β-TC-6 cells were seeded onto 6-well plate at a density of 4×10^5 cells/well and cultured for two days in normoxic conditions. After two days, medium was changed with fresh medium containing 19,5 µg/ml of BA and NaB and the cells were cultured in hypoxic conditions for two days. SOD enzyme activity of β-TC-6 cells in hypoxic conditions has been determined by OxiSelect™ Superoxide Dismutase Activity Assay kit (#STA-340, CELL BIOBLAS, INC., SAN DIEGO, USA) according to the manufacturer's instructions. Cells were washed with ice-

cold Phosphate-buffered saline(PBS). The cells were harvested into one ml of cold one x Lysis Buffer. The cells were lysed with sonication(Ultrasonic Cleaner, Lab Companion, Jeio Tech Co. Ltd, South Korea). The cells were centrifuged at 12000 x g for 10 mins and supernatant was collected. Samples, including a blank, in a 96-well microtiter plate were given in Table 2.4. 10µl of pre-diluted 1X Xanthine Oxidase Solution was added to each well. Wells were mixed and incubated for one hour at 37 °C. Absorbance was measured at 490nm by using an ELISA plate reader (Biotek, Winooski, VT).

Table 2.4. Microtiter plate plan for SOD enzyme activity

Componenet	**Blank**	**Sample**
SOD Sample	0 µl	X µl
Xanthine Solution	0 µl	Y µl
Chromagen Solution	5 µl	5 µl
10X SOD Assay Buffer	10 µl	10 µl
DI Water	70 µl	70-(X+Y) µl
Total	**90 µl**	**90 µl**

2.1.6. Glutathione Peroxidase (GPx) Enzyme Activity

The activity of GPx enzyme, which has low expression in type I diabetes, was determined in the current study. The mechanism of action and roles of GPx enzyme were given in different studies[217,218]. β-TC-6 cells were seeded onto 6-well plate at a density of 4×10^5 cells/well and cultured for two days in normoxic conditions. After two days, medium was replaced with fresh medium containing 19,5 µg/ml of BA and NaB and the cells were cultured in hypoxic conditions. Glutathione peroxidase (GPx) enzyme activity of boron derivatives-treated β-TC-6 cells was measured by Glutathione Peroxidase Activity Kit (#ADI-900-158, Enzo Life Sciences, USA)**.** Briefly, the cells were washed with 1X PBS. The cells were harvested into five ml of ice-cold 1X PBS and centrifuged at 400 x g for 10 min at 4 °C. Supernatant was discarded. The cell pellet was resuspended in

1 ml ice-cold 1X PBS and transferred to a pre-chilled 1.5 ml microtube. It was centrifuged at 10,000 x g for 12 seconds at 4°C. Supernatant was discarded and then the cell pellet was resuspended in five pellet volume of ice-cold 1X Assay Buffer. The cell suspension was incubated on ice, with periodic vortexing, for 30 min. The disrupted cell suspension was centrifuged at 10,000 x g for 10-20 minutes at 4°C to remove insoluble material. The supernatant was recovered to a fresh tube pre-chilled on ice. Plate reader was set up to measure absorbance at 340 nm every one minute. A 10 second orbital shake prior to the initial read was included. Reactions in a 96-well plate (per well) were set up. The experimental conditions were given in Table 2.5. 20 µL of Cumene Hydroperoxide was added to each well using a multichannel pipettor to initiate the reaction. Absorbance was measured at 340nm every 1 minute by using an ELISA plate reader for 10-15 mins (Biotek, Winooski, VT).

Table 2.5. Experimental conditions for GPx Enzyme Activity

1X Assay Buffer	140 µl
10X Reaction Mix	20 µl
Glutathione Peroxidase, sample or control	20 µl

2.1.7. Insulin Secretion Assay

The importance of insulin hormone was discussed by Wilcox et al. [219]. In type I diabetes, β-cells of the pancreas are destroyed by the immune system of the host body leading to the hyperglycemia. Patients with type I diabetes must receive exogenous insulin regularly. β-TC-6 cells were seeded onto 6-well plate at a density of 4×10^5 cells/well and cultured for two days in normoxic conditions. After two days, medium was changed with fresh medium containing 19,5 µg/ml of BA and NaB and the cells were cultured in hypoxic conditions. Serum insulin level was analyzed by using a commercial ELISA kit (#EZRMI-13K, Rat/Mouse Insulin 96-Well Plate Assay Kit, Millipore, MA, USA). Briefly, 10X Wash Buffer diluted with de-ionized water. Strips assembled in an empty plate holder and wells were washed three times with 300µl of diluted buffer. Wash Buffer was decanted and the residual amount was removed from each well by inverting the plate.

10µl of Assay Buffer was added to each of the blank and sample wells. 10µl of Matrix Solution was added to the Blank, Standard and control wells. 10µl of Rat Insulin standard was added in order of ascending concentration to the appropriate wells. 10 µL QC1 and 10 µL QC2 were added to the appropriate wells. Afterwards, 80µl Detection Antibody was added to all wells. The plate was covered with plate sealer and incubated at room temperature for two hour. After two hour, plate sealer was removed and solutions from the plate were taken away. Each well was washed with 300µl diluted Wash Buffer. 100µl Enzyme Solution was added to each well and plate was incubated with shaking at room temperature for 30 min on the microtiter plate shaker (Titramax 100, Heidolph, Germany). Sealer was taken back and all fluid from wells were removed. The wells were washed with 300µl of Wash Buffer for 6 times and fluids were decanted and all residual buffer was removed. 100µl of Substrate Solution was added to each well. Plate was covered with sealer and shaked in the plate shaker for 15-20 min. Lastly, sealer was removed and 100µl of Stop Solution was added to each well. Plate was shaked by hand to ensure complete mixing of solutions in all wells. Absorbance was measured at 450-590nm by using an ELISA plate reader.

2.2. STATISTICAL ANALYSIS

One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was conducted for multiple comparisons of data using GraphPad Prism statistical software 5.0 (GraphPad Software, La Jolla, CA, USA). The values of $P<0.05$ were considered statistically significant.

3. RESULTS

3.1. CELL VIABILITY

3.1.1. Normoxia

Cytotoxic and effective concentrations of boron derivatives were determined by using MTS-assay in normoxic conditions. Various concentrations of boron derivatives (625µg/ml, 312.5µg/ml, 156.25µg/ml, 78.1µg/ml, 39.06µg/ml, 19.5µg/ml, 9.76µg/ml, 4.88µg/ml, 2.44µg/ml, 1.22µg/ml and 600ng/ml) were used to detect the effect of boron chemicals on the cell viability of pancreatic β-TC-6 cells. The results revealed that concentrations between 156.25µg/ml and 2.44µg/ml for BA and NaB significantly increased the cell viability rate of β-TC-6 cells at day one and day two in normoxic conditions. The best results were obtained from 19,5µg/ml of NaB and BA application. While 19,5 µg/ml of NaB treatment for 24h resulted in an approximately 74 per cent increase in the cell number, 19,5µg/ml BA treatment for 24h resulted in an approximately 110 per cent increase compared to control group. On the second day of 19,5µg/ml of NaB application, 27 per cent increase was obtained. On the second day of 19,5µg/ml of BA application, 54 per cent increase was detected in the cell viability compared to control group, indicating the proliferative effect of boron compounds on pancreatic β-TC-6 cells. However, boron treatment for both 19,5µg/ml of BA and NaB on the third day restricted viable β-TC-6 cells compared to control group. There was not any statistically significant difference between any experimental groups for 19,5µg/ml of BA and NaB at day three. Figure 3.1. shows the effect of various concentrations of NaB on pancreatic β-TC-6 cells in normoxic conditions ($P<0.05$).

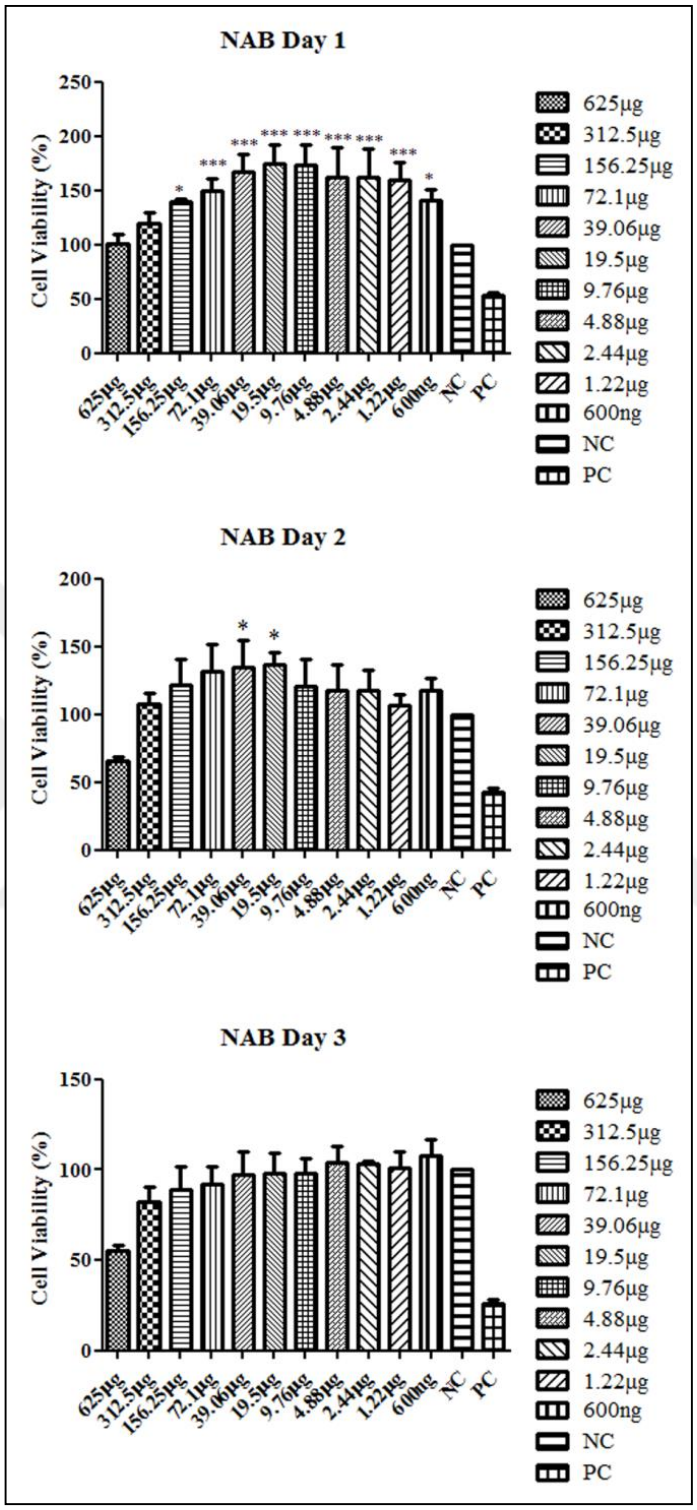

Figure 3.1. The effect of various concentrations of NaB on pancreatic β-TC-6 cells in normoxic conditions. Notes: Negative Control: Complete growth medium, Positive Control: 20 per cent DMSO containing growth medium. *P<0.05. NC was accepted as 100 per cent for each day.

The results revealed that cell number of β-TC-6 cells were enhanced by boron treatment at the concentration ranging from 156.25µg/ml and 2.44µg/ml for BA and NaB at day one and two. An average increase of 74 per cent and 110 per cent in viable cell ratios were obtained at day one for both 19,5µg/ml concentrations of NaB and BA, respectively. Boron application showed analogous effect with an average 27 per cent and 54 per cent increase in the cell viability rate for 19,5µg/ml concentration of NaB and BA at day two, respectively. Therefore, the concentration of 19,5µg/ml for both NaB and BA was selected for further experiments. The effect of various concentrations of BA on pancreatic β-TC-6 cells in normoxic conditions was shown in figure 3.2.

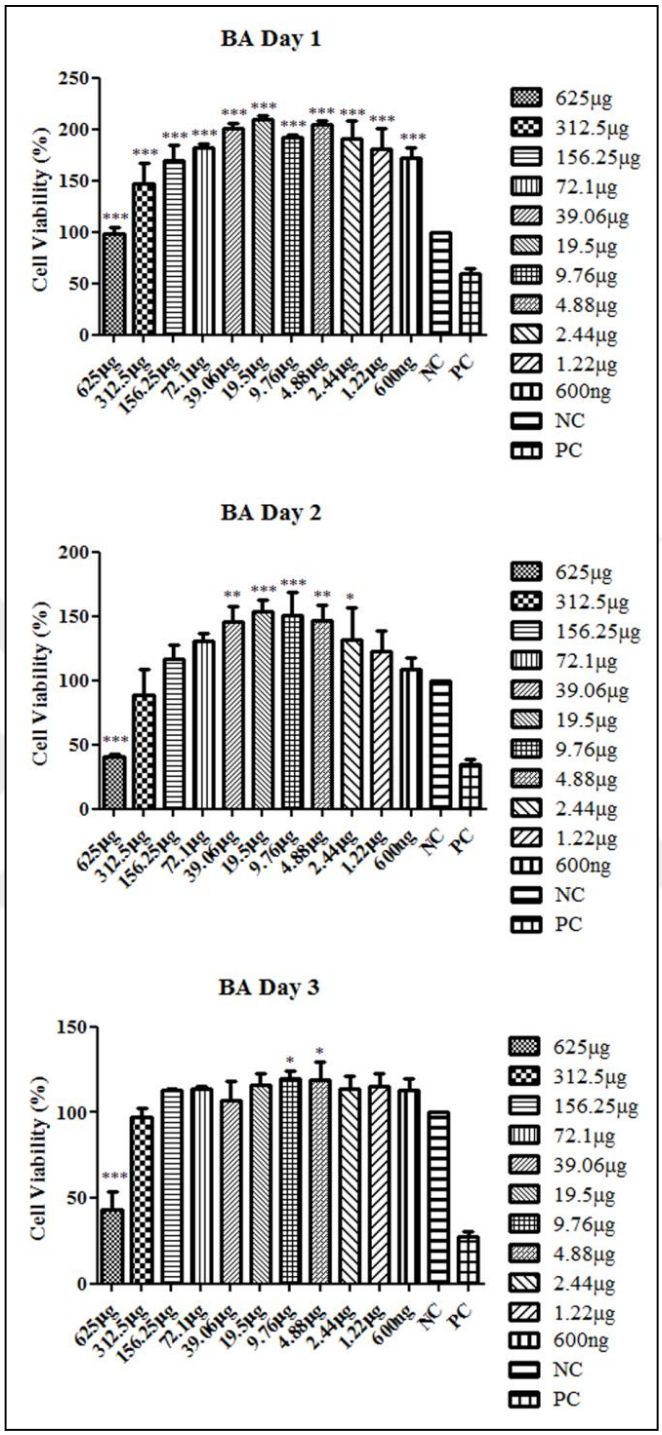

Figure 3.2. The effect of various concentrations of BA on pancreatic β-TC-6 cells in normoxic conditions. Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20 per cent DMSO containing growth medium) *P<0.05. NC was accepted as 100 per cent for each day.

3.1.2. Hypoxia

The most effective concentration of BA and NaB was determined as 19.5µg/ml in normoxic conditions and this concentration was also used to see the effect of BA and NaB in hypoxic conditions for two days. Although NaB treatment resulted in an approximately 11 per cent increase, BA treatment provided 22 per cent increase in the cell viability in the first day compared to control group in hypoxic conditions. On the second day of application, an increase of 18 per cent was observed in NaB-treated β-TC-6 cells. BA-treated β-TC-6 cells showed 33 per cent increase compared to control group in hypoxic conditions after two days incubation period. The results demonstrated that boron-derivatives inhibited the cell death caused by hypoxic environment. Boric acid was more effective in inhibiting the cell death compared to NaB in hypoxic conditions at day one and two. Figure 3.3. indicates the effect of 19,5µg/ml of BA and NaB on pancreatic β-TC-6 cells on hypoxic conditions.

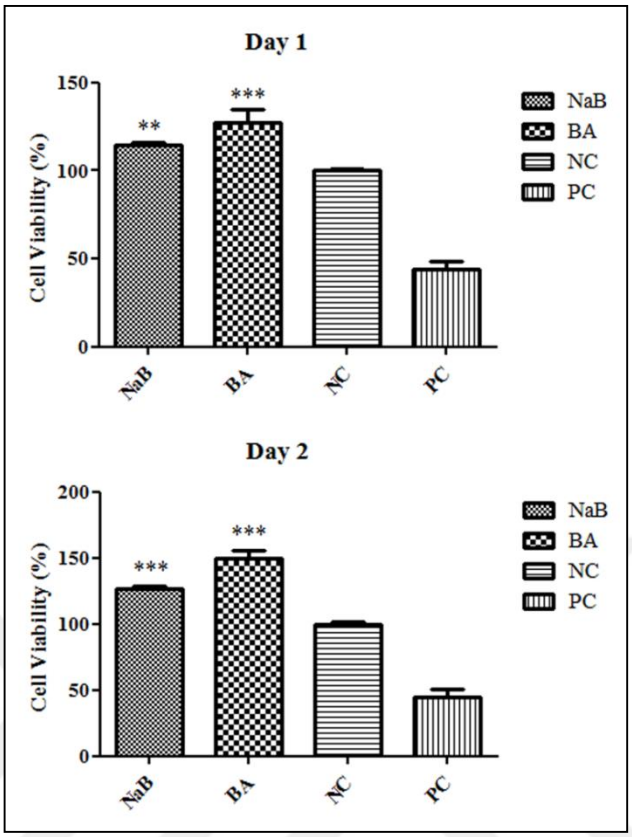

Figure 3.3. The effect of 19,5µg/ml of BA and NaB on pancreatic β-TC-6 cells on hypoxic conditions. Notes: NC: Negative Control (complete growth medium), PC: Positive Control (20 per cent DMSO containing growth medium) *P<0.05. NC was accepted as 100 per cent for each day.

3.2.RT-PCR ANALYSIS

To examine potential protective mechanism of boron-derivatives, pancreatic β-TC-6 cells were treated with NaB and BA at aferomentioned concentration (19,5µg/ml) for 24 and 48h. Afterwards, apoptosis-associated gene expression levels were evaluated using RT-PCR analysis. Interestingly, NaB and BA have different effects on apoptotic markers. It was found that the expression of caspase 3 gene, one of the essential mediators for apoptosis, was found to be significantly lower in BA treated β-TC-6 cells at day one as well as p53, whereas NaB treatment did not cause a significant change in mRNA expressions of caspase 3 and p53 genes at day one. BA and NaB treatment significantly decreased caspase 3 gene expression level at day two. However, 19,5µl of BA and NaB

treatment for two days did not result in any decrease in p53 expression levels in hypoxic conditions (Figure 3.4)

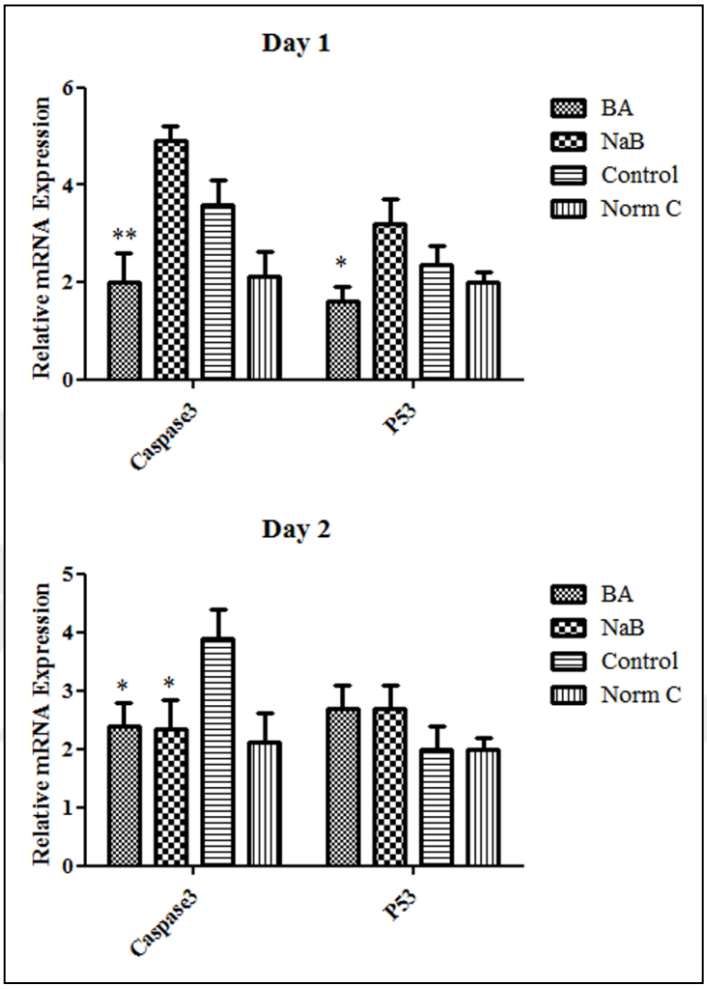

Figure 3.4. The effect of BA and NaB on mRNA expression levels of apoptosis associated genes in mouse pancreatic β-TC-6 cells. Abbreviations: BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

Antiapoptotic genes such as Bcl-2 and Akt expressions were also evaluated to identify the effect of boron-derivatives on pancreatic β-TC-6 cells in hypoxic conditions. The results displayed that 19,5µg/ml of NaB and BA concentrations significantly increased on both Bcl-2 and Akt gene expressions in hypoxic conditions at day one and 2 (Figure 3.5)

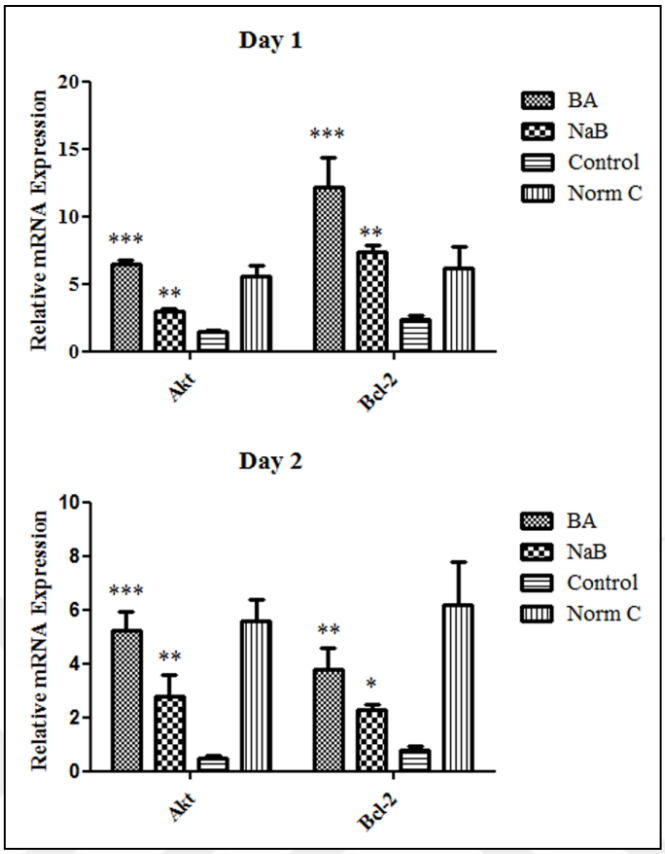

Figure 3.5. The effect of BA and NaB on mRNA expression levels of anti-apoptotic genes in mouse pancreatic β-TC-6 cells. Abbreviations: Akt: Protein kinase B, Bcl-2: B-cell lymphoma 2 BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

The effect of boron derivatives on transforming growth factor beta 1 (TGFβ-1) and The interleukin 4 (IL-4), involving in the prevention of type I diabetes, gene level in β-TC-6 cells were analyzed. The results showed that aforementioned concentrations of BA and NaB have similiar effect on pancreatic β-TC-6 cells for TGFβ-1 and IL-4 genes in hypoxic conditions at day one and two. It was found that BA and NaB significantly enhanced mRNA expression level of TGFβ-1 and IL-4 genes (Figure 3.6).

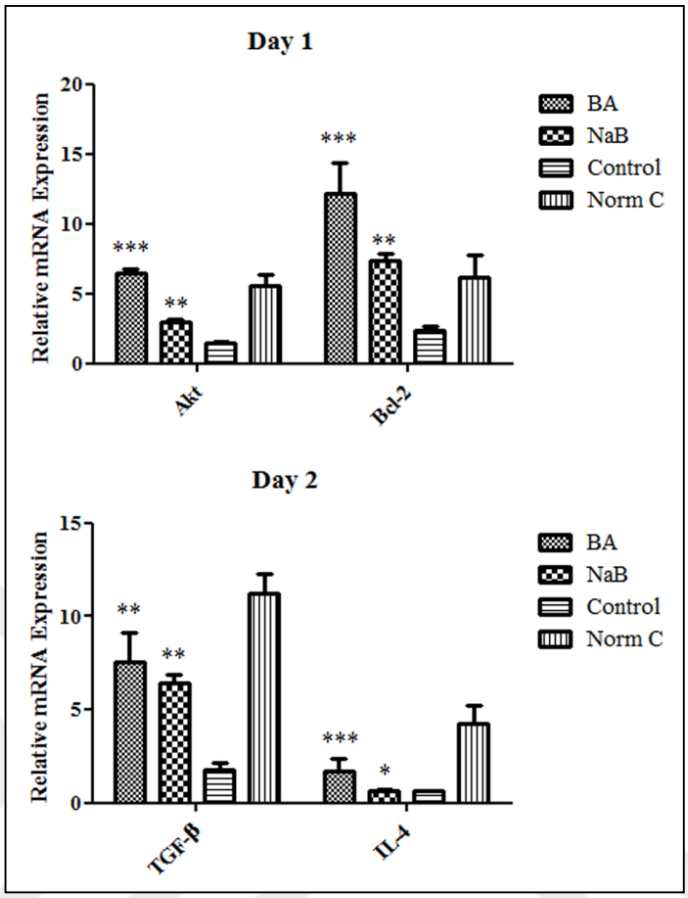

Figure 3.6. The effect of BA and NaB on mRNA expression levels of anti-inflammatory cytokines in mouse pancreatic β-TC-6 cells. Abbreviations: TGFβ-1: Transforming growth factor beta 1, IL-4: The interleukin 4, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

To examine the potential role of boron derivatives on insulin production mechanism, expression levels of two important genes were evaluated by RT-PCR. Pancreatic β-TC-6 cells were treated with 19,5µg/ml of BA and NaB and insulin production-associated gene expression levels were analyzed. The results indicated that mRNA expression levels of preproinsulin and insulin genes, involved in insulin biosynthesis mechanisms, increased by the treatment of boron derivatives in hypoxic conditions at day one and two compared to untreated cells. The effect of boron derivatives on PPI genes were shown in Figure 3.7. Increase in insulin gene expression level was given in Figure 3.8.

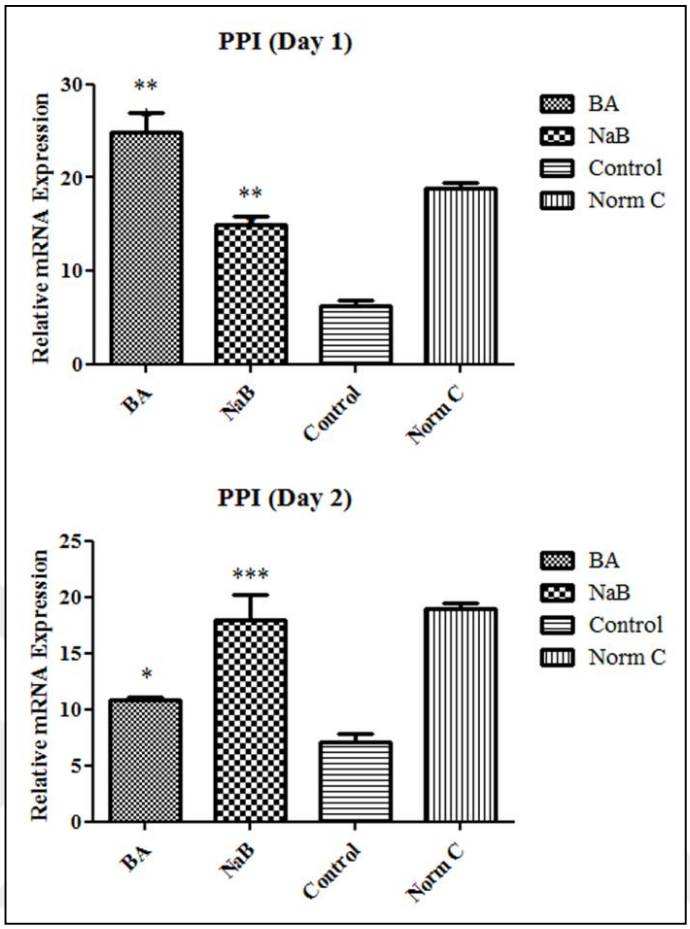

Figure 3.7. The effect of BA and NaB on mRNA expression levels of preproinsulin gene in mouse pancreatic β-TC-6 cells. Abbreviations: PPI: Preproinsulin, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

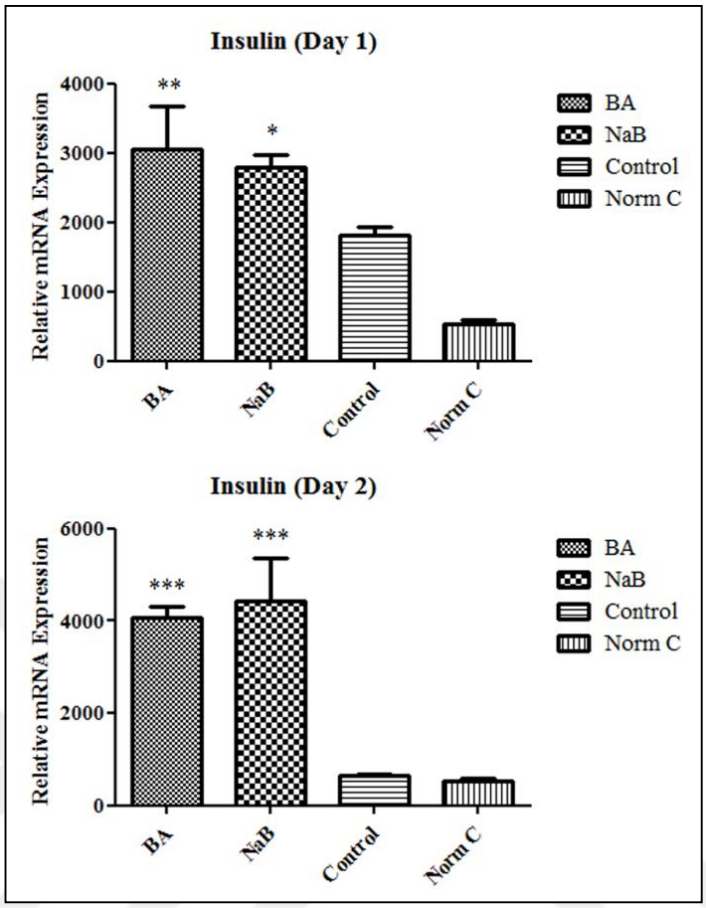

Figure 3.8. The effect of BA and NaB on mRNA expression levels of insulin gene in mouse pancreatic β-TC-6 cells. Abbreviations: BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

The expression level of pancreatic and duodenal homeobox-1 (Pdx-1), necessary for β-cell maturation and pancratic development, was also checked. The results showed that NaB and BA treatment significantly triggered Pdx-1 mRNA expression in hypoxic conditions at day one and two compared to control group. While BA is more effective than NaB at day one, NaB increased Pdx-1 gene expression more at day two compared to BA treatment (Figure 3.9).

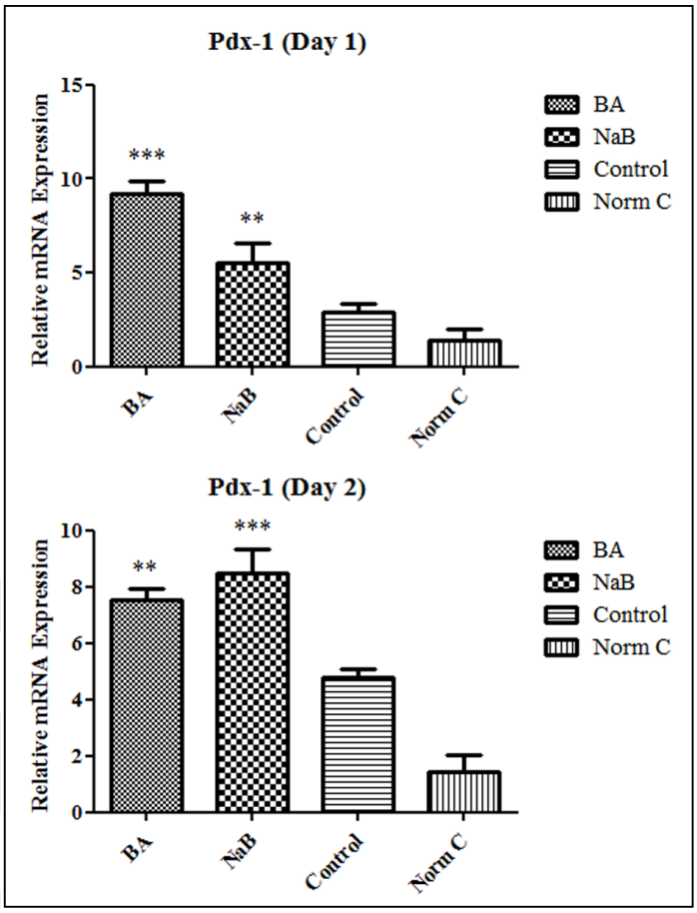

Figure 3.9. The effect of BA and NaB on mRNA expression levels of Pdx-1 gene in mouse pancreatic β-TC-6 cells. Abbreviations: Pdx-1: Pancreatic and duodenal homeobox-1, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

Glucokinase (Gck), an important gene for glucose metabolism, was investigated. The results displayed that mRNA expression level of Gck gene, a crucial glucose sensor for β cells, dramatically increased in pancreatic β-TC-6 cells treated 19,5µg/ml of NaB and BA in hypoxic conditions at day one and two (Figure 3.10).

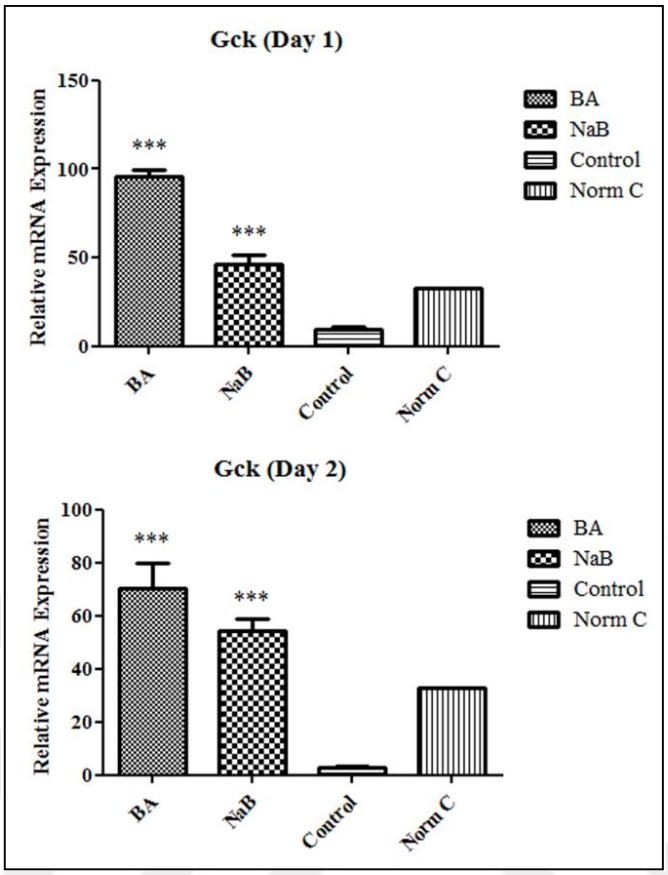

Figure 3.10. The effect of BA and NaB on mRNA expression levels of Gck gene in mouse pancreatic β-TC-6 cells. Abbreviations: Gck: Glucokinase, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

Glucose transporter 2 (Glut2), the only glucose transporter in β-cells, is an important key for glucose-stimuli insulin secretion. Glut2 expression level in pancreatic β-TC-6 cells in hypoxic conditions was evaluated. 19,5µg/ml of BA and NaB treatment for 24 and 48h resulted in a significant increase in Glut2 expression level (Figure 3.11).

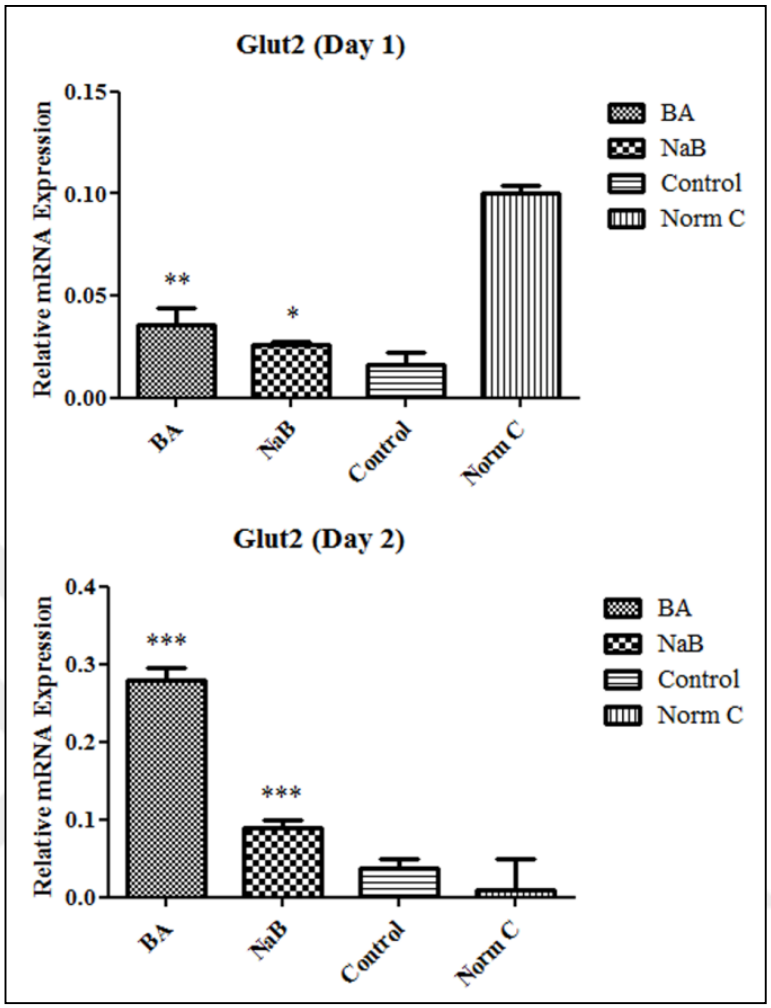

Figure 3.11. The effect of BA and NaB on mRNA expression levels of Glut2 gene in mouse pancreatic β-TC-6 cells. Abbreviations: Glut2: Glucose Transporter 2, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

mRNA expression levels of two genes from superoxide dismutase family were investigated. SOD1 (CuZn-SOD), cupper-zinc superoxide dismutase, and SOD2 (manganese superoxide dismutase (Mn-SOD) genes, playing important roles to neutralize superoxide anion radicals derived from extracellular stimulants, have low expression level in type I diabetes. β-TC-6 cells were treated with 19,5µg/ml NaB and BA in hypoxic conditions for two days. The results showed that BA treatment did not result in any

increase in SOD1 gene expressions at day one and two, whereas NaB treatment significantly increased SOD1 gene expression at day one in hypoxic conditions. Aforementioned concentrations of NaB and BA treatment for 24h did not cause any change in SOD2 gene expression. However, both NaB and BA treatment at aforementioned concentrations for 48h significantly increased SOD2 gene expression in hypoxic conditions at day two compared to control group (Figure 3.12).

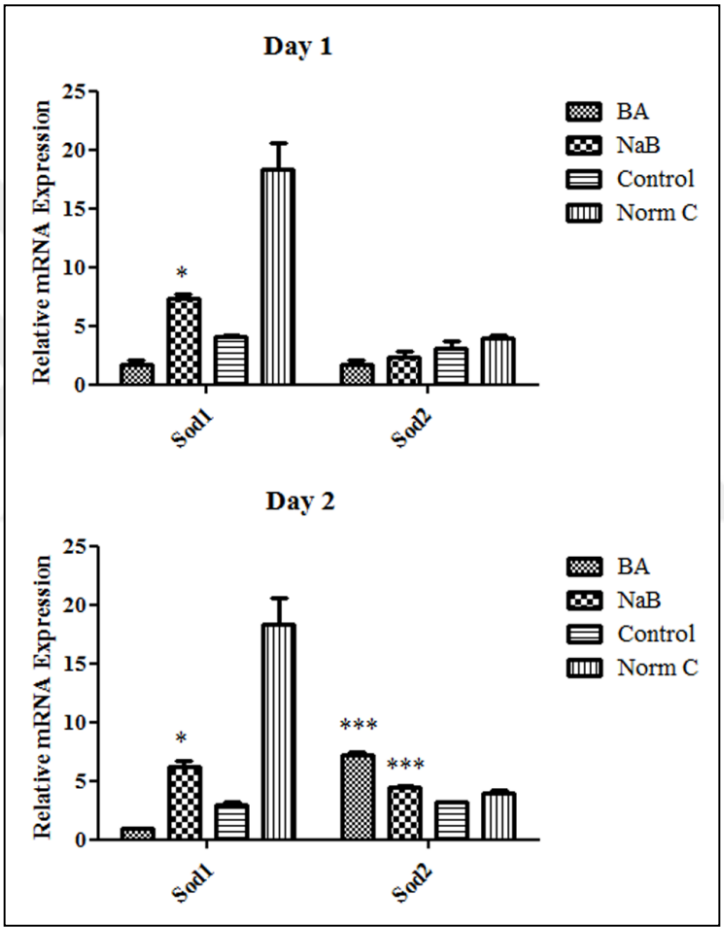

Figure 3.12. The effect of BA and NaB on mRNA expression levels of SOD1 and SOD2 antioxidant enzyme genes in mouse pancreatic β-TC-6 cells. Abbreviations: Sod1: Copper–zinc superoxide dismutase, Sod2: Manganese superoxide dismutase, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

3.3.ANNEXIN V APOPTOSIS DETECTION

In the current study, pancreatic β-TC-6 cells were treated with 19,5µg/ml BA and NaB to investigate whether the boron derivatives cause programed cell death in hypoxic conditions. Apoptosis was measured in cells treated with BA and NaB containing medium or normal growth medium by AnnexinV labelling. Figure 3.13 indicates Annexin V/Propidium Iodide staining analysis of growth medium treated β-TC-6 cells in normoxic conditions at day one (Figure 3.13 a) and in hypoxic conditions (Figure 3.13 b). As expected, the apoptotic and necrotic cell fractions in growth medium treated cell were low. In addition, flow cytometer analysis of BA and NaB treated β-TC-6 cells showed that the treatment of BA (Figure 3.13 c) and NaB (Figure 3.13 d) have not ended up with apoptosis in hypoxic conditions at day one.

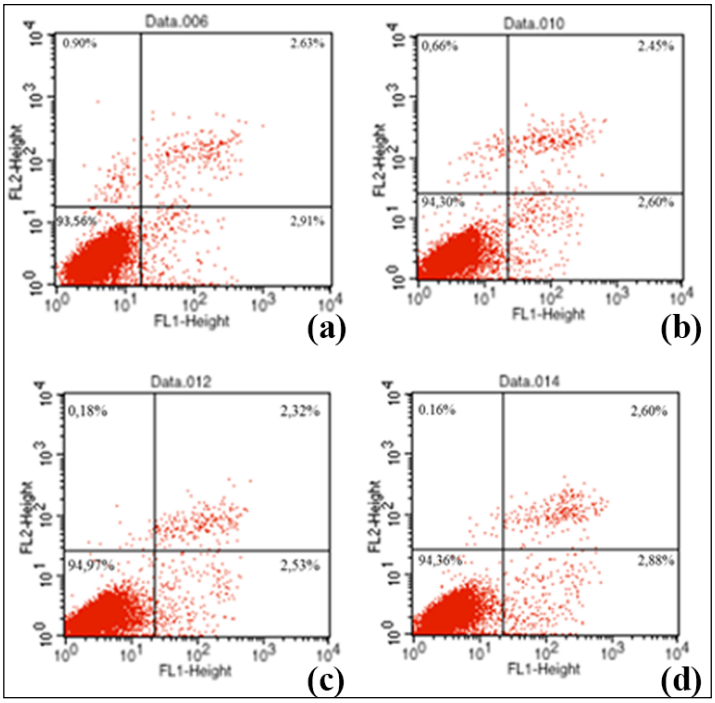

Figure 3.13. FACS results of the Annexin V-FITC and PI assay at day one. Abbreviations: a: Growth medium treated β-TC-6 cells in normoxic conditions, b: Growth medium treated β-TC-6 cells in hypoxic conditions, c: 19,5µg/ml of Boric Acid treated β-TC-6 cells in hypoxic conditions, d: 19,5µg/ml of NaB (Sodium pentaborate pentahydrate) treated β-TC-6 cells in hypoxic conditions.

Figure 3.14 a and 3.14 b demonstrated Annexin V/Propidium Iodide staining results of growth medium treated β-TC-6 cells in normoxic and in hypoxic conditions at day two, respectively. The results revealed that while there was low number of apoptotic (1.28 per cent) and necrotic (1.01 per cent) cells in growth medium treated cells under normoxic conditions at the end of 48 h incubation period, hypoxia increased apoptosis (16.16 per cent). However, there was not a significant difference in necrotic cell percentage (0.29 per cent). Moreover, NaB and BA treated β-TC-6 cells did not result any significant difference in apoptosis or necrosis as early apoptotic cell percentages were 15.28 and 15.86, and necrotic cell proportions were 0.14 per cent and 0.48 per cent for NaB and BA treatments, respectively (Figure 3.14 c and d). In overall evaluation of data proved that while BA and NaB treatments did not decrease apoptosis in hypoxic conditions, and they also did not augment apoptotic cell numbers with respect to only growth medium treated groups.

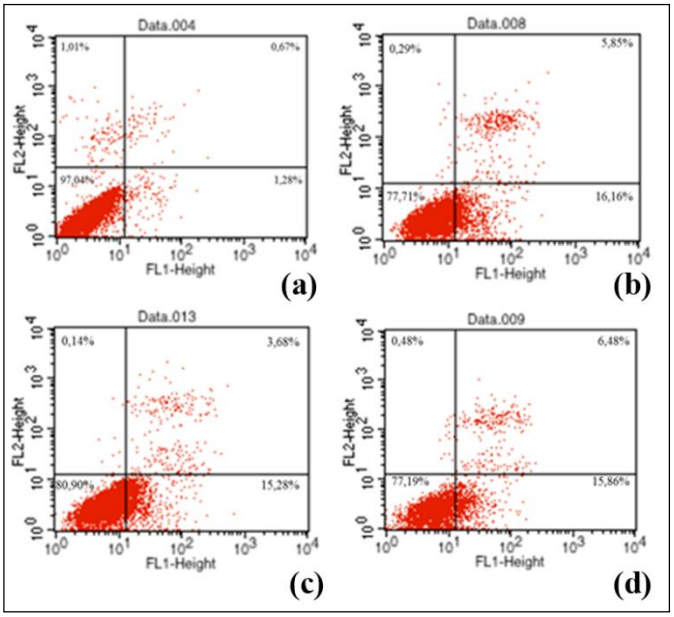

Figure 3.14. FACS results of the Annexin V-FITC and PI assay at day two. Abbreviations: a: Growth medium treated β-TC-6 cells in normoxic conditions, b: Growth medium treated β-TC-6 cells in hypoxic conditions, c: 19,5µg/ml of NaB (Sodium pentaborate pentahydrate) treated β-TC-6 cells in hypoxic conditions, d: 19,5µg/ml of BA (Boric Acid) treated β-TC-6 cells in hypoxic conditions.

3.4.SOD ENZYME ACTIVITY

Superoxide dismutase is one of the most crucial antioxidant enzymes and catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen. Pancreatic β-TC-6 cells were treated with 19,5µg/ml of NaB and BA for 24 and 48h and SOD enzyme activity was evaluated. The results displayed that SOD enzyme activity in 19,5µg/ml of NaB and BA treated β-TC-6 cells significantly increased at day one and two in hypoxic conditions. As a result of the application, SOD enzyme activity of β-TC-6 cells cultured in hypoxic conditions became similiar to β-TC-6 cells cultured in normoxic conditions. Figure 3.15 depicts that the highest increase in SOD enzyme activity was obtained at day one and two for both NaB and BA treated β-TC-6 cells.

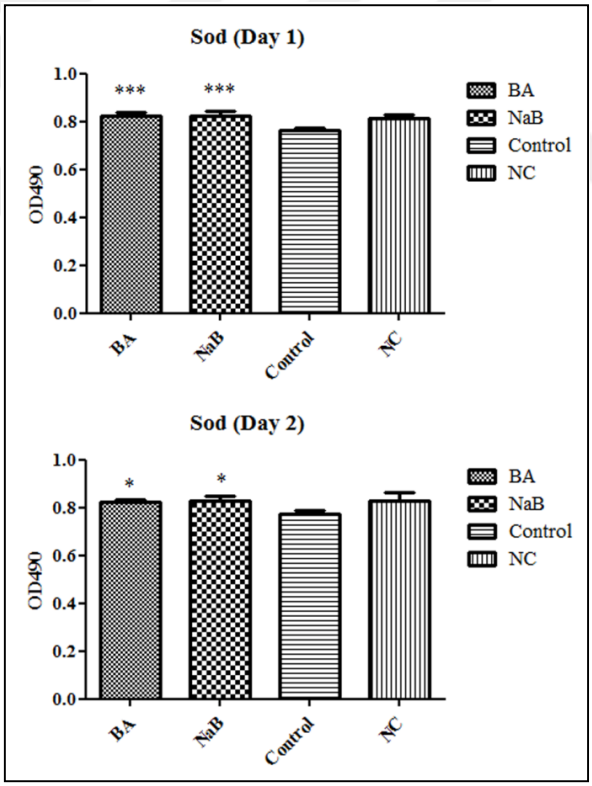

Figure 3.15. The effect of BA and NaB on SOD antioxidant enzyme activity of mouse pancreatic β-TC-6 cells. Abbreviations: Sod: Superoxide Dismutase, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, NC: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

3.5.GLUTATHIONE PEROXIDASE (GPx) ENZYME ACTIVITY

Glutathione Peroxidase (GPx) is an antioxidant enzyme and protects the organism from oxidative stress. Hydrogen Peroxide is converted to oxygen (O_2) and water (H_2O) by glutathione peroxidase. Pancreatic β-TC-6 cells were treated with 19,5µg/ml of NaB and BA in hypoxic conditions for 24 and 48h and GPx enzyme activity was measured. The results revealed that NaB treatment significantly increased GPx enzyme activity in hypoxic conditions at day one and two. In addition, BA treatment did not result in any significant change at day one. However, GPx antioxidant enzyme activity significantly increased as a result of BA treatment at day two (Figure 3.16).

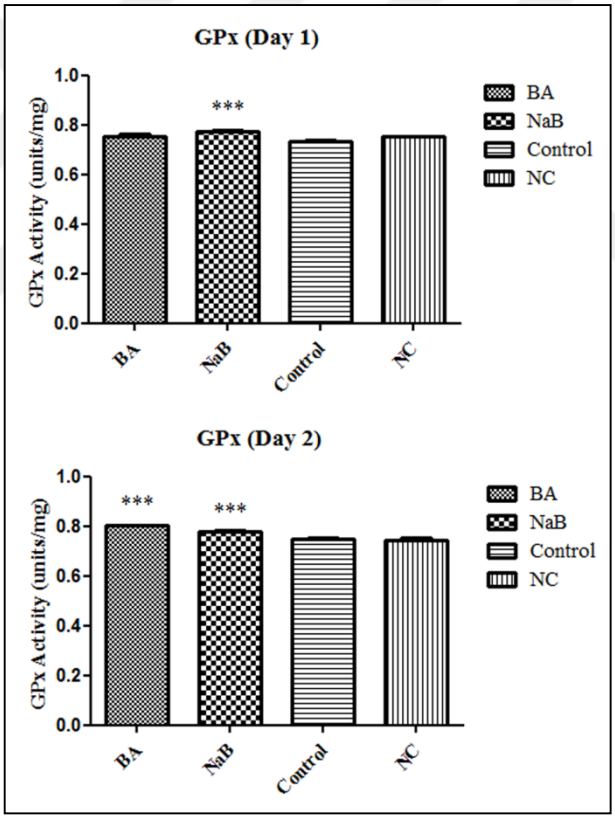

Figure 3.16. The effect of BA and NaB on GPx antioxidant enzyme activity of mouse pancreatic β-TC-6 cells. Abbreviations: GPx: Glutathione Peroxidase, BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, NC: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

3.6.INSULIN SECRETION ASSAY

Insulin, one of the most important hormones regulating glucose metabolism, is the key factor in diabetes treatment. In type I diabetes, insulin secretion is very low. Basically, panceatic β-TC-6 cells were treated with aferomentioned concentrations of BA and NaB and insulin secretion from culture media was evaluated by rat/mouse insulin ELISA assay. The results revealed that NaB and BA treatment for 24 and 48h in hypoxic conditions increased insulin secretion compared to untreated control group. In the first day of boron treatment, it was found that BA was slightly more effective than NaB. At the end of 48h in hypoxic conditions, it was observed that NaB is more effective than BA treatment. The results are given in Figure 3.17.

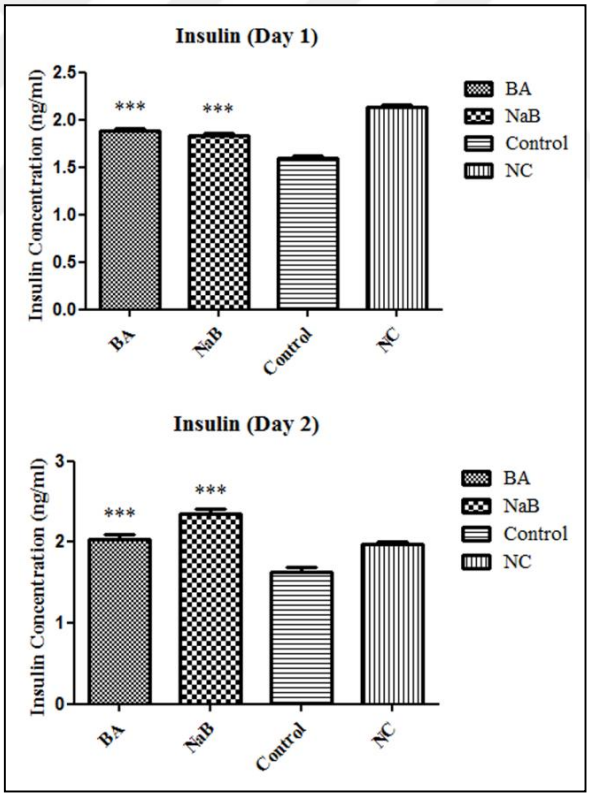

Figure 3.17. The effect of BA and NaB on insulin secretion of mouse pancreatic β-TC-6 cells. Abbreviations: BA: Boric Acid, NaB: Sodium pentaborate pentahydrate, Control: Growth medium treated β-TC-6 cells in hypoxic conditions, Norm C: Growth medium treated β-TC-6 cells in normoxic conditions. Notes: Results were analyzed by one-way ANOVA and Tukey's post hoc test, *P<0.05.

4.DISCUSSION

Diabetes Mellitus is a worlwide disease. It is characterized by hyperglycemia caused by problems in insulin action, insulin secretion or both. Many organs in the human body such as the kidneys, heart and eyes can be seriously affected by diabetes. The common symptoms are loss of weight, thirst, blurring of vision and polyuria. Diabetes have long-term effects decrasing quality of life. Retinopathy, nephropathy, neuropathy and ketoacidosis are just a few of the long-term effects of diabetes. Patients have high risk of cerebrovascular and cardiovascular diseases. Physical inactivity, obesity and overeating are the main reasons of diabetes. Uncontrollable risk factors such as age, family history and genetics can also be involved in the development of the disease.

Although studies on the classification of diabetes are still a matter of debate, there are basically four types of diabetes. Type I diabetes, type II diabetes, gestational diabetes and other specific types of diabetes.

The destruction of pancreatic β-cells producing insulin is the main characteristic of type I diabetes. β-cells are destroyed by the immune system of the body[222]. Between 70-90 per cent of pancreatic β-cells of the patients with type I diabetes are lost. People having type I diabetes become addicted to daily insulin uptake[223]. Diagnosis of the diabetes is performed by measuring blood glucose level. To measure the level of blood sugar, fasting plasma glucose test (FPGT) is generally conducted. Fasting blood glucose level above 125 mg/dL is accepted as diabetes. Type I diabetes accounts for five-10 per cent of all cases of diabetes. In 2012, 29.1 million Americans have diabetes mellitus and 1.25 million of them have type I diabetes. Diabetes is the 8th fatal disease causing 1,5 million deaths in 2012 around the world. In Turkey, acccording to a report published by Republic of Turkey Ministry of Health, there are 7.043.290 diabetic patients in 2013.

Several therapies are available for treating diabetes. The most important one is daily insulin uptake. Patients with type I diabetes use recombinant human insulin. Another available treatment for type I diabetes is pancreas and/or islet transplantation[224,225]. Scientists also use some chemicals or additional substances to recruit the complications of diabetes. Studies are mostly focused on increasing antioxidant enzyme activity and cell survival[226].

Therefore, identification of new substances for diabetes treatment is of great interest. It was shown that boron derivatives increased hormone action, carbohydrate metabolism and the production of RNA in plants. Apart from plants, boron derivatives are also effective on animals and humans. Boron deprivation affects metabolic and physiological systems such as mineral, bone, lipid and energy metabolism of the mammalian body[227]. Boron derivatives are effective on the cell viability and it was shown by our group previously[228]. Experimental studies revealed that boron increases antioxidant enzyme activity[194] and also has effects on IL-1β which induces apoptosis of pancreatic β-cells[205]. Based on this, boron derivatives were used to promote the survival and the maintenance of β-cells.

In the current study, boron derivatives were investigated for their effects on type I diabetes mellitus using *in vitro* (BA and NaB). Apart from their proliferative properties, effects on pro-apoptotic and anti-apoptotic markers, genes including glucose and insulin metabolisms were evaluated. Moreover, antioxidant enzymes activities were also investigated by RT-PCR and ELISA assays. The effect of boron derivatives on insulin secretion was analyzed.

It is a well know issue that boron increases the cell viability[228] and promotes cell proliferation. Therefore, boron derivatives were tried on pancreatic β-TC-6 cells. Different concentrations ranging from 625µg/ml to 600 ng/ml were prepared and applied on the cell. The effect of boron derivatives on the cell viability of β-TC-6 cells were evaluated for 24, 48 and 72h in normoxic conditions. Among prepared concentrations, 19,5µg/ml of BA treatment for 24h showed the highest increase with 110 per cent compared to untreated control group. For NaB, the highest increase (74 per cent) was also obtained from 19,5µg/ml of NaB concentration at the end of 24h. 19,5µg/ml of BA treated pancreatic β-cells showed the highest increase with 54 per cent at the end of 48h. NaB treatment for 48h resulted in an increase of 27,5 per cent in cell viability compared to untreated β-cells. By the end of the third day, 19,5µg/ml of BA treatment did not significantly increase cell viability compared to untreated control group. The highest cell viability was obtained from 9,765µg/ml of BA treatment with lower rate. The application of NaB to β-cells did not promote cell viability at any concentration. The results revealed that the highest increase in cell viability was obtained from 19,5µg/ml of BA and NaB concentration at day one and two. 19,5µg/ml concentration was chosen for further *in vitro* experiments.

There are two ways to mimic *in vitro* model of type I diabetes. The first one is the addition of hydrogen peroxide (H_2O_2) into the cells. The second one is the incubation of β-cells in a hypoxic chamber[226,229]. As mentioned before, pancreatic β-cells were incubated in hypoxic conditions for 24 and 48h to mimic *in vitro* model of type I diabetes mellitus. β-TC-6 cells were treated with 19,5µg/ml of BA and NaB and their effects on cell viability were examined. The results showed that both BA and NaB are effective to increase cell viability in hypoxic conditions compared to untreated control group. At the end of the first day, the highest increase in cell viability was obtained from BA treatment with 22 per cent. Meanwhile, NaB treatment provided an increase with 11,5 per cent compared to untreated control group. At the end of the second day, both BA and NaB treatment maintained the increase in cell viability. While BA treatment resulted in an increase with 32 per cent, NaB treatment provided 17 per cent increase in cell viability compared to control group. The main problem in type I diabetes is the cell death resulting from apoptosis or oxidative stress. Boron treatments in hypoxic conditions revealed that boron derivatives significantly increased cell viability of β-cells. In the literature, there are studies showing the effect of boron on cell viability. In 2014, it was shown that boron treatment increased the cell viability of mesenchymal stem cells[261]. Moreover, it has been shown that BA and NaB treatment increased cell viability of dermal cells such as L-929, HUVEC and human fibroblast[228]. However, the effect of boron compounds on cell viability of pancreatic β cells were not shown. As mentioned in the literature, boron compounds and their salts serve as buffering agent in various application. Boron is also tend to create complex structure with organic molecules. This features of boron compounds might be the reason of increase in cell viability.

Molecular mechanism of boron has not been fully understood yet. To examine that, pro and anti apoptotic markers, genes which affect the preservation of pancreatic β-cells, genes related to glucose and insulin metabolisms were investigated. It was reported that apoptosis is the main cause of β-cell death in diabetes mellitus[230]. Caspases, produced in the cells as inactive zymogen, are mainly classified as initiator or effector caspases. Caspase 3, one of the effector caspases, is necessary for the cascade of apoptosis[231,232]. Therefore, the effect of boron derivatives on caspase 3 gene was evaluated. The results showed that BA treatment significantly decreased caspase 3 gene expression at day one and two compared to untreated control group. Interestingly, caspase 3 gene expression was found high in NaB

treated β-TC-6 cells at day one. However, the expression of caspase 3 gene significantly reduced at day two. The results indicate that boron treatment is effective on caspase 3 gene expression and reduced its level.

The p53 tumor suppressor, named "the guardian of the genome", is very important for apoptosis and can be activated by DNA damage and hypoxia[233]. It is involved in apoptosis, DNA repair mechanism and cell cycle regulation[234]. In the current study, p53 was also examined to understand apoptotic state of hypoxia-incuced β-TC-6 cells. The results indicated that BA treatment for 24h significantly reduced p53 mRNA expression level compared to untreated control group. However, BA application for 48h did not result in the decrease in mRNA expression level of p53 gene. Unexpectedly, p53 gene expression level of NaB-treated β-TC-6 cells was high at day one and two. Active form of p53 gene does not always lead to apoptosis because there are p53-dependent and p53-indepedent pathways of apoptosis[235]. As shown, BA and NaB differently affected the expression levels of caspase 3 and p53 genes. This might be because of the difference in pH during dissolution.

Antiapoptotic genes such as B-cell lymphoma 2 (Bcl-2) and serine/threonine-specific protein kinase (Akt) were also checked to observe whether boron derivatives prevents apoptosis. Bcl-2 is a protein family that regulates apoptosis by incuding or inhibiting. Some members of the family are antiapoptotic (Bcl-2, Bcl-XL and Mcl-1), some members are proapoptotic (Bax, Bak and Bok)[236]. RT-PCR results showed that BA and NaB treatment significantly increased Bcl-2 gene expression at day one and two compared to untreated control group. mRNA expression level of Akt gene was similar to Bcl-2. The results indicated that NaB and BA treatment significantly increased Akt gene expression level at day one and two. The results of antiapoptotic gene expression levels revealed that boron derivatives can be used to increase cell viability in type I diabetes.

Cytokines, nonstructural and small proteins, are secreted by nonimmune and immune cells. They have roles in apoptosis, differentitation and cell proliferation. They play a fundemental role in the regulation of T cells differentiation[237]. They are divided into two basic categories. Proinflammatory and antiinflammatory cytokines. Transforming growth factor beta 1 (TGFβ-1) and the interleukin 4 (IL-4) are two of anti-inflammatory cytokines. In the literatures, there are studies showing the effect of TGFβ-1 and IL-4 on type I diabetes. It was shown that TGFβ-1 takes in charge in the prevention of type I

diabetes. IL-4 has also protective effects on type I diabetes as well as TGFβ-1[238,239]. In the present study, the expression of TGFβ-1 and IL-4 were evaluated by RT-PCR analysis. The results indicated that BA treatment for 24 and 48h significantly increased TGFβ-1 gene expression at day one and two compared to untreated control group. BA treatment for 24 and 48h significantly increased IL-4 gene expression level at day one and two compared to control group. NaB treatment for 24h also increased IL-4 expression at day one. However, no increase in IL-4 expression at day two was observed.

The main problem in type I diabetes is the destruction of insulin-producing β-cells. People having type I diabetes substantially lose their pancreatic β-cells. Insulin production levels for this type of patients is very low. The dependence of insulin in these patients is unavoidable. Chromosome 11 in humans codes insulin gene. Insulin is synthesized in the β cells of the pancreatic islets of Langherhans. First, the insulin mRNA produced and then translated into preproinsulin (PPI) which is single chain precursor of insulin. Removal of single peptide of preproinsulin in the endoplasmic reticulum generates proinsulin. Proinsulin is transferred by secretory vesicles from from the RER (rough endoplasmic reticulum) to the golgi apparatus for further processes[219]. In this study, the effect of boron derivatives on insulin and its precursor preproinsulin gene was analyzed by RT-PCR. Our results suggested that BA and NaB treatment were effective on both PPI and insulin gene expressions. PPI gene expression level in BA treated β-TC-6 cells were found to be significantly higher at day one and two compared to untreated control group. Similar results were obtained for NaB treated β-cells. PPI gene expression significantly increased as a result of the application of NaB at day one and two compared to control group. BA and NaB treatment resulted in a significant increase in the expression level of insulin gene. In the first day, BA and NaB treated β-cells showed significantly higher expression at day one. Similarly, BA and NaB application significantly increased the mRNA expression level of insulin gene at day two.

Glucose is the main stimuli for insulin production and secretion. Augmented glucose level triggers glucose-mediated insulin secretion from β-cells. Glucokinase (Gck), an enzyme serving as glucose sensor in β-cells of the pancreas, senses the entry of glucose into the β-cells and facilitates phosphorylation of glucose to glucose-6-phosphate[219]. Insulin secretion is stimulated by glucose-6-phosphate. Therefore, the expression of Gck gene is crucial in type I diabetes[240]. In this study, Gck gene expression level was examined. BA

and NaB treatment significantly increased Gck gene expression level at day one and two compared to untreated control group.

Pancreatic and duodenal homeobox-1 (Pdx1) is a transcription factor necessary for β-cell maturation and pancreatic development[241]. During embryogenesis, Pdx-1 expression can be firstly detected in the embryo at day 8.5[242]. Pdx-1 also plays a role in the maintenance of healthy β-cell function[243]. The expression level of Pdx-1 gene in BA and NaB treated β-cells was evaluated. Our results displayed that both BA and NaB treatment significantly increased the expression level of Pdx-1 gene at day one and two compared to untreated control group.

Glucose is transported by glucose transporter family which have 14 members. Glucose transporter 2 (Glut2), the only glucose transporter in β-cells, in mainly expressed in pancreatic β-cells. Increased blood glucose level induces insulin secretion. In β-cells, the absence of Glut2 inhibits insulin secretion stimulated by glucose[244]. In the current study, the expression level of Glut2 gene was investigated. BA and NaB treatment for 24h did not result in any increase in mRNA expression level of Glut2 gene. However, BA and NaB treatment significantly increased Glut2 gene expression level at day two compared to untreated control group. Glucose, transported by Glut2, is very important for insulin secretion. The effect of boron compounds on Glut2 gene was shown for the first time in the current study. Our results revealed that boron compounds significantly increased Glut2 gene expression level. Boron compounds might be used in glucose-stimuli insulin secretion studies.

One of the biggest problems in diabetes mellitus is oxidative stress. Production of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) is defined as oxidative stress. Moreover, it refers the imbalance between production of reactive species and antioxidant enzyme defence mechanism[245]. The main source of free radicals is glucose oxidation. The superoxide anion radicals, which is highly toxic for the organism, is produced by aerobic respiration and has to be degraded by antioxidant enzymes[246]. The superoxide anion radicals produced by glucose oxidation undergo dismutation. Thus, hydrogen peroxide(H_2O_2) is formed. Catalase or glutathione peroxidase degrades H_2O_2. Otherwise, highly reactive hydroxyl radicals are formed as mentioned before. Superoxide radical is converted to H_2O_2 by SOD enzyme. Afterwards, toxic H_2O_2 is detoxified to H_2O and O_2 by glutathione peroxidase[97]. In diabetes, β-cells of the pancreas are very sensitive

to free radicals such as ROS and RNS because the expression of antioxidant enzymes such as SOD, glutathione peroxidase (GPX) and catalase are limited compared to liver tissue[247]. SODs have three isoforms. Copper–zinc superoxide dismutase (Cu/ZnSOD, SOD1), manganese superoxide dismutase(Mn-SOD, SOD2), and extracellular superoxide dismutase(ECSOD, SOD3). SOD1 was the first characterized superoxide dismutase enzyme found in cytoplasm, microsomes and nucleus. It was shown that transgenic mice in which SOD1 is overexpressed are resistant to STZ-induced diabetes[248]. Among SOD isoforms, SOD2 is the only SOD that was proved to be crucial for the survival of oxygen-breathing organisms[249]. It was shown that the impairment in mitochondrial ROS production was achieved by the overexpression of SOD2 gene[97]. Furthermore, pancreatic islets overexpressed with SOD2 gene was able to increase insulin secretion and cell viability [250]. SOD enzymes are so important in type I diabetes. Therefore, the mRNA expression levels of SOD1 and SOD2 gene were investigated in the current study. SOD1 gene expression level significantly increased in NaB treated β-TC-6 cells at day one and two compared to untreated control group. However, BA treatment did not result in any increase at day one and 2. The reason of SOD1 inactivity may be its localization in the cell or molecular structure of BA. It must be illuminated by other studies. SOD2 gene expression level was examined by RT-PCR analysis. The results showed that BA and NaB are not effective on SOD2 gene expression level at day one. Nevertheless, SOD2 gene expression level significantly increased in NaB and BA treated β-cells at day two. Total SOD enzyme activity of NaB and BA treated β-cells was measured. The results indicated that SOD enzyme activity of NaB and BA treated β-cells was found significantly higher than untreated control group. All results related to SOD enzyme indicated that BA and NaB are effective on SOD and induce its activation. The activation of glutathione peroxidase enzyme converting H_2O_2 to H_2O and O_2 was examined. Expectedly, BA and NaB treatment increased GPx enzyme activity at day one and two compared to untreated control group. Our results related to antioxidant enzymes indicated NaB and BA treatment increase antioxidant enzymes activity. As discussed before, antioxidant enzyme activity in type I diabetes is very low. Boron derivatives can be used to increase their activities.

Our gene expression data indicated that BA and NaB treatment increased preproinsulin and insulin gene expression level. In the next step, Insulin level from serum was measured. In a manner consistent with preproinsulin and insulin gene expression results, BA and NaB

treatment significantly increased insulin level in plasma at day one and two compared to untreated control group. Both BA and NaB seem more effective at day two. In the literature, there are studies showing the effect of boron compounds on calcium metabolism. In glucose-stimuli insulin secretion, calcium and calcium channels have crucial importance. Boron derivatives might affect calcium and calcium channels and trigger insulin secretion from β cells.

Exact molecular mechanism of boron is unknown in both *in vitro* and *in vivo*. Moreover, boron derivatives have not used in type I diabetes. Therefore, the effect of boron derivatives on type I diabetes was investigated in the current study. Firstly, the effective concentration of boron derivatives was determined in normoxic conditions. Different concentrations of boron derivatives were increased the cell viability but the highest increase was obtained from 19,5µg/ml. Hypoxic conditions were used to mimic type I diabetes in *in vitro*. Our results displayed that boron derivatives (BA and NaB) also have a major effect on cell viability of β-cells of the pancreas in hypoxic conditions. In the next step, boron derivatives were investigated for their effects in gene level. Pro-apoptotic and anti-apoptotic markers, genes related to insulin production mechanism, some antioxidant enzymes, and genes having important roles in the development of diabetes were examined by RT-PCR analysis. Basically, BA and NaB were found positively effective on genes previously mentioned. However, NaB did not decrease the expression level of pro-apoptotic genes p53 and BAX. It was observed that NaB and BA treated β-TC-6 cells did not undergo apoptosis according to apoptosis&necrosis analysis. As known, the expression of p53 and BAX are parallel to each other. Increase in p53 gene expression did not lead to apoptosis. p53 independent pathway of apoptosis was indicated in the literature[251]. Antioxidant enzyme activity of β-TC-6 cells were induced by boron derivatives treatment in hypoxic conditions. In the next step, insulin level in serum was measured and an increase was obtained from both BA and NaB treated cells. Antioxidant enzyme activity, which is a big problem in type I diabetes, was evaluated. Results showed that boron treatment increased antioxidant enzyme activity such as SOD and GPx. BA significantly increased cell viability, positively affected gene expression levels, increased antioxidant enzyme activity and insulin secretion level *in vitro*.

Exact molecular mechanism of boron is unknown and it’s the major limitation of boron studies. In the current study, the effect of boron compounds on β cell viability, gene

expression levels of antioxidant ezymes, and insulin biosynthesis, beta cell maturation and glucose metabolism were shown for the first time as well as insulin secretion and antioxidant enzyme activities. Our results revealed that boron derivatives might be used in type I diabetes studies. For further investigations, the effect of boron derivatives could be tried in different β cell lines and more parameters could be investigated. Moreover, animal studies should be performed to obtain positive effects of boron derivatives *in vivo* diabetic studies. In the future, boron-containing drugs could be developed to decrease insulin dependency of type I diabetes patients.

Evidences in the present and previous studies suggested that boron is playing an important role in mineral and hormonal metabolisms, cell membrane functions and enzyme reactions[179, 180, 181]. In addition, boron coumpounds have been shown to have antioxidant, antimicrobial, anticancer, anti-osteoporotic and anti-inflammatory activities both *in vitro* and *in vivo* studies[194, 228, 268]. Data from deficiency studies showed that low amounts of boron intake is necessary for some animals in order to complete their life cycle. All of these findings may indicate that boron should be recognized as an essential element, which could make positive contributions to human and animal health.Dietary intake and/or new drug formulations containing boron may have an important role in reducing plasma lipids, and treatments of a number of clinacally important diseases such as cancers, metabolic, orthopedic, neurodegenerative, autoimmune, infectious, coronary heart and endocrine system diseases in the future.

5. CONCLUSION

In today's world, there are millions of people are suffering from diabetes mellitus. New treatment strategies should be improved such as insulin uptake and pancreas transplantation. Insulin dependency of patients with diabetes mellitus should be decreased or completely solved. The data gathered from this study obviously indicated that boron derivatives increased cell viability, antioxidant enzyme activity, insulin release of of β-TC-6 cells. Moreover, the expression levels of genes taking charge in apoptosis, insulin production, antioxidant enzymes and some cytokines having protective effects in type I diabetes were positively affected by boron derivatives treatment. Overall data suggest that boron derivatives potentially provide a new treatment option for the treatment of type I diabetes mellitus. However, further studies are required to investigate signaling pathways, which boron is involved, to understand molecular mechanisms. To increase the effect of boron derivates, they could be combined with pluronic triblock copolymers widely used in biological applications as vehicles.

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