Exercise Training Prevents and Protects Streptozotocin-Induced Oxidative Stress and β-Cell Damage in Rat Pancreas

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COSKUN, O., OCAKCI, A., BAYRAKTAROGLU, T. and KANTER, M. *Exercise Training Prevents and Protects Streptozotocin-Induced Oxidative Stress and β-Cell Damage in Rat Pancreas.* Tohoku J. Exp. Med., 2004, **203** (3), 145-154 —— The aim of the present study was the evaluation of possible protective effects of exercise against β-cell damage in streptozotocin (STZ)-induced diabetes in rats. The animals were divided into five groups: the control group, the STZ-induced diabetes group, the STZ-induced diabetes and light-intensity exercise group, the STZ-induced diabetes and moderate-intensity exercise group, and the STZ-induced diabetes and heavy-intensity exercise group. Animals in the exercise groups were made to swim one of three exercise protocols once a day for 12 consecutive weeks. STZ was injected intraperitoneally at a single dose of 50 mg/kg for diabetes induction. Exercise training was continued for 4 weeks prior to STZ administration; these applications were continued end of the study (for 12 weeks). Erythrocyte and pancreatic tissue malondialdehyde (MDA) levels and serum nitric oxide (NO) concentration were measured. Moreover glutathione peroxidase (GSHPx), superoxide dismutase (SOD) and catalase (CAT) were also measured in pancreatic homogenates. Pancreatic β-cells were examined by immunohistochemical methods. STZ increased lipid peroxidation and decreased the antioxidant enzyme activity significantly. Exercise, especially moderate-intensity exercise has shown protective effect probably through decreasing lipid peroxidation and increasing antioxidant enzyme activity. Islet cell degeneration and weak insulin immunohistochemical staining were observed in STZ induced diabetic rats. Increased intensity of staining for insulin and preservation of β-cell numbers were apparent in the exercise-applied diabetic rats. Interestingly, the best result was obtained from moderate-intensity exercise. These findings suggest that exercise has a therapeutic and/or protective effect in diabetes by decreasing oxidative stress and preservation of pancreatic β-cell integrity. ———— exercise; diabetes; streptozotocin; β-cell; oxidative stress

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Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defective insulin secretion or resistance to insulin action, or both (Braun et al. 1995). Oxidative stress may constitute a focal point for multiple therapeutic interventions, and for therapeutic synergy. Hyperglycemia is thought to promote oxidative stress through both non-enzymatic and enzymatic mechanisms. Oxidative stress plays an important role in the etiology of diabetes and diabetic complications (Baynes and Thorpe 1996). Ihara et al. (1999) examined oxidative stress markers in diabetic rats and found increased reactive oxygen species (ROS) in pancreatic islets.

STZ, an antibiotic produced by Streptomyces achromogenes, is the most commonly used agent in experimental diabetes (Rakieten et al. 1963). The mechanism by which STZ destroys β-cells of the pancreas and induces hyperglycemia is still unclear. Many actions have been attributed to STZ that are similar to what has been described for the diabetogenic action of alloxan that include damage to pancreatic β-cell membrane (Bhattacharya 1954) and to depletion of intracellular nicotinamide dinucleotide in islet cells (Schein et al. 1973). In addition, STZ has been shown to induce DNA strand breaks and methylation in pancreatic islet cells (Matkovics et al. 1997-1998).

Nitric oxide synthase is present in pancreatic β-cells and may be involved in the release of insulin under normal physiological conditions (Moncada et al. 1991). However, findings suggest that induction of nitric oxide formation may play a role in the destruction of the β-cells during the development of type 1 diabetes (Corbbet et al. 1993). Antioxidant defense mechanisms are important for protection of cells and tissues from oxidative damage and consist of non-enzymatic antioxidants and antioxidant enzymes, which includes SOD, CAT, GPx, and GSH (Nadler and Winter 1996). Reduced antioxidant levels as a result of increased free radical production in experimental diabetes have been reported by many authors (Grankvist et al. 1981; Kanter et al. 2003a). Regular physical exercise has been reported to be effective in the prevention and delay of onset of non-insulin-dependent diabetes, increases insulin sensitivity, and ameliorates glucose metabolism (Derouich and Boutayeb 2002). Eight weeks treadmill training increased the endurance, favorably decreased lipid peroxidation as measured by thiobarbituric acid reactive substances (Gul et al. 2002).

The present study was undertaken to determine whether the pancreas was subjected to oxidative damage during diabetes as well as to examine the accompanying changes in antioxidant status in order to understand its role in the pathogenesis of the disease. In addition, we explored whether exercise protects pancreatic β-cells STZ-induced in diabetic rats.

MATERIALS AND METHODS

Animals

Fifty healthy male Wistar albino rats, weighing 200-250 g, and averaging 16 weeks old were utilized in this study. They were housed in macrolon cages under standard laboratory conditions (light period 7.00 a.m. to 7.00 p.m., 21 ±2°C, relative humidity 55%). The animals were given standard rat pellets (Murat animal food product Co., Ankara, Turkey) and tap water *ad libitum*. The rats were randomly allotted into one of five experimental groups: A (control), B (diabetic), C (diabetic light-intensity exercise training), D (diabetic moderate-intensity exercise training) and E (diabetic heavy-intensity exercise training), each containing 10 animals. B, C, D and E groups received STZ (Sigma, St. Louis, MO, USA). Diabetes was induced in both groups by a single intraperitoneal (i.p) injection of STZ (50 mg/kg of body weight, freshly dissolved in five mmol/liter citrate buffer, pH 4.5) (Kanter et al. 2003b). Control group was injected with the same volume of isotonic NaCl as the diabetic groups received. Four weeks prior to induction of diabetes, C group was applied swimming for 5 minutes, D group was applied same exercise for

10 minutes and E group was swam for 15 minutes once a day. These applications were continued daily until the end of the study (for 12 weeks). Light, moderate and heavy exercises were chosen on the basis of a previous studies (Braun et al. 1995; Nikolovski et al. 1996; Bräu et al. 1997; Derouich and Boutayeb 2002; Gul et al. 2002; Henriksen 2002). Because rats are natural swimmers, exercise protocols based on swimming are widely used, the intensity of the exercise being determined by the amount of lead weight attached to the tail (Nikolovski et al. 1996; Bräu et al. 1997). All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health.

Exercise protocol

Immediately before swimming, each animal was weighed, and a lead weight equivalent to 9% of body mass was attached to the base of the tail to exercise each rat at near-maximal intensity. The 9% lead weight consisted of three lead weights of equivalent mass, each suspended by a small hook to a hook holder attached with a rubber band to the animal's tail. Swimming took place in a 30-cm-diameter plastic tank filled with water (48 cm deep) at 34°C. To exercise the rats to near exhaustion, the size of the weight was progressively reduced on each occasion by one-third, as the animals tired, until two-thirds of the weight was removed. Each weight was removed by pulling it off the hook holder while the animal was swimming. Exhaustion was defined as that point at which the animal could not remain at the water surface (Nikolovski et al. 1996; Bräu et al. 1997).

Biochemical assays

Blood glucose and serum insulin analysis. Two days after STZ treatment, development of diabetes was confirmed by measuring blood glucose levels in a tail vein blood samples. Rats with blood glucose levels of 250 mg/100 ml or higher were considered to be diabetic. Plasma glucose levels in control animals remained normal for the duration of the study. The diabetes mellitus was confirmed by Ames One Touch Glucometer (LifeScan, Johnson and Johnson, New Brunswick, NJ, USA).

At the end of the experiment rats in all groups were starved overnight for 12 h, and sacrificed under chloralhydrate (6 ml of 7% chloralhydrate kg; Sigma, St. Louis, MO, USA) anaesthesia. Blood samples were collected by cardiac puncture using heparinised syringe. Serum glucose was determined by hexokinase method with reagents from Boehringer (Mannheim, Germany), and insulin was determined by a double-antibody radioimmunoassay (RIA) kit (Amersham Radiochemical Centre, Bucks, UK) (Kanter et al. 2003b). All serum insulin samples were assayed by RIA using crystalline rat insulin as standard. Free and antibody-bound insulin was separated by precipitation with ethanol (Heding 1966). ^{125}I-labeled insulin was supplied by Eurodiagnostica (Malmö, Sweden).

Lipid peroxides and nitric oxide

Lipid peroxide levels were measured in plasma, hemolysate and tissue homogenates as thiobarbituric acid reactivity. The product of the reaction between malondialdehyde and thiobarbituric acid was measured as described by Thayer (1984). Nitric oxide was determined in plasma as nitrite concentration after reduction of nitrate to nitrite. The reaction was performed at 22°C for 20 minutes and the absorbance at 546 nm was measured using $NaNO_3$ solution as standard (Ding et al. 1988).

SOD, CAT and GSH-Px activities

Tissue SOD and GSH-Px activities were measured by using Ransod and Ransel (Randox Laboratories GmbH, Deutschland) commercial kits, respectively by using a Shimadzu UV-1601 spectrophotometer. Tissue CAT activity was determined according to Aebi's method (Aebi 1974).

Histopathological procedures

Pancreatic tissues were harvested from the sacrificed animals, and the fragments from tissues were fixed in 10% neutral formaline solution, embedded in paraffin and then, stained with haematoxylin and eosin.

Immunohistochemical procedures

Harvested pancreatic tissues fixed in 10% neutral buffered formaline, were embedded in paraffin and sectioned at 5 μm thickness. Immunohistochemical reactions were performed using the ABC technique according to Hsu et al. (1981). Specific monoclonal mouse antisera against human insulin protein (Zymed 18-0066, CA, USA) were applied at a dilution of 1:50. The procedure was performed by the following steps: 1) inhibition of endogenous peroxidase activity was inhibited with 3% H_2O_2 in distilled water for 30 minutes, 2) washed in tap water for 30 minutes and in distilled water for 10 minutes, 3) blocked of non-specific binding of antibodies by incubation with normal goat serum (DAKO × 0907) with PBS, diluted 1:4, 4) incubation with monoclonal mouse antisera against human insulin protein, diluted 1:400 for 2 hours, then at room temperature, 5) washed in PBS 3×3 minutes, 6) incubation with biotinylated anti-mouse IgG (DAKO LSAB 2 Kit, 7) washed in PBS 3×3 minutes, 8) incubation with ABC complex (DAKO LSAB 2 Kit), 9) washed in PBS 3×3 minutes, 10) peroxidase detection using AEC, 11) washed in tap water for 10 minutes, dehydration, 12) nuclei stained with hematoxylin, and 13) sections mounted in DAKO faramount. Preparations were evaluated by a bright field microscope and were photographed (Nikon Optiphot 2, Tokyo).

Ten Langerhans islets from each rat, thus hundred islets for each group, were chosen randomly. Semiquantitative intensity of staining for insulin and β-cell numbers in pancreatic islets were scored as − (absence of β-cell), ± (trace of β-cell), + (low), ++ (medium) and +++ (high).

Statistical analysis

The data were expressed as mean ± standard deviation (s.e.) and analysed using repeated measures of variance. Tukey test was used to test for differences among means when ANOVA indicated a significant ($p<0.05$) and ($p<0.001$) F ratio. For the analysis of the immunohistochemical data, a nonparametric test (Kruskal-Wallis) was used. Differences were considered statistically significant when the p value was <0.05.

RESULTS

Body weight, blood glucose levels and serum insulin levels of experimental animals are shown in Table 1. The baseline weight of the rats at the beginning of the study was similar in all groups.

TABLE 1. *Body weight, serum glucose levels and serum insulin levels of A (control), B (diabetic), C (diabetic light-intensity exercise training), D (diabetic moderate-intensity exercise training) and E (diabetic heavy-intensity exercise training)*

	n	A	B	C	D	E
Initial body weight (g)	10	226±12[a]	228± 8[a]	226±12[a]	226±12[a]	224± 6[a]
Final body weight (g)	10	228± 7[a]	198± 7[b]	219± 6[a]	221± 7[a]	218± 9[a]
Initial serum glucose (mg/dl)	10	102± 8[a]	105± 8[a]	101± 8[a]	102± 8[a]	103± 6[a]
Final serum glucose (mg/dl)	10	99± 6[a]	322±11[b]	232±14[c]	203±14	192±14[e]
Initial Serum insulin (mU/l)	10	57± 5[a]	56± 5[a]	58± 4[a]	57± 6[a]	59± 5[a]
Final Serum insulin (mU/l)	10	56± 4[a]	16± 3[b]	25± 4[c]	34± 4[c]	28± 4[c]

Values are expressed as mean ± s.d., and n=10 for all groups.
[a] Insignificant difference ($p>0.05$) between all groups.
[b,c,d,e] Significant difference ($p<0.05$) between treatments and control or other treatments group.

TABLE 2. *Erythrocyte MDA (µmol/g Hb), serum NO (µmol/l) and pancreatic tissue MDA (µmol/g protein), SOD (U/mg protein), GSH-Px (U/mg protein) and CAT (k/mg protein) levels of all groups. A (control), B (diabetic), C (diabetic light-intensity exercise training), D (diabetic moderate-intensity exercise training) and E (diabetic heavy-intensity exercise training)*

Parameters	A	B	C	D	E
Erythrocyte MDA	8.92±1.07[a]	15.23±2.03[b]	11.48±1.24[c]	10.44±1.23[c]	11.22±1.24[c]
Tissue MDA	96±11[a]	134±22[b]	119±14[c]	108±13[c]	109±14[c]
Serum NO	4.22±0.68[a]	7.42±1.22[b]	4.96±0.94[a]	4.76±0.84[a]	5.12±0.84[a]
Tissue SOD	22.88±9.61[a]	12.42±1.32[b]	16.08±2.24[c]	19.18±9.51[c]	18.14±9.52[c]
Tissue GSH-Px	0.38±0.03[a]	0.23±0.03[b]	0.29±0.03[c]	0.30±0.03[c]	0.31±0.03[c]
Tissue CAT	0.36±0.02[a]	0.21±0.02[b]	0.26±0.02[c]	0.27±0.02[c]	0.26±0.02[c]

Values are expressed as mean±S.D., and *n*=10 for all groups.
[a,b,c] Means in the same row with different superscripts significantly differ ($p<0.05$).

At the end of the treatment (after 4 weeks), diabetic animals in group B presented weight loss. The initial and final body weights were not significantly different in control rats and exercise-trained diabetic rats.

Biochemical findings

The diabetic animals exhibited consistently hyperglycemia. Exercise training caused a decrease ($p<0.05$) in the elevated serum glucose and an increase ($p<0.05$) in the lowered serum insulin (Table 1) concentrations in STZ-induced diabetic rats with the elapse of the experiment.

Erythrocyte MDA, serum NO and pancreatic tissue MDA, SOD, GSH-Px and CAT levels were presented in Table 2. Erythrocyte MDA, serum NO concentration and pancreatic tissue MDA significantly ($p<0.05$) increased and also the antioxidant levels significantly decreased ($p<0.05$) in diabetic group. Exercise training significantly ($p<0.05$) decreased the elevated MDA and NO and also increased ($p<0.05$) the reduced antioxidant enzyme activities in diabetic light-intensity exercise training group, diabetic moderate-intensity exercise training group and diabetic heavy-intensity exercise training group.

Histopathological findings

The histology of pancreatic islet cells was normal in control group. In immunohistochemical staining of the pancreatic tissues in control group was observed strong insulin antigen positivity in the β-cells of the islets (Fig. 1a).

In diabetic rats with no treatment, the most consistent findings in the histologic sections of pancreatic tissues stained with haematoxylin and eosin were the degenerative and necrotic changes, and shrunken in the islets of Langerhans. The nucleus of necrotic cells indicated either pyknosis or marginal hyperchromasia. There was mostly hydropic degeneration and degranulation in the cytoplasm of the degenerative and necrotic cells, while the some of cells with pyknotic nucleus had the dark eosinophilic cytoplasm. In immuno-histochemical staining of the pancreatic tissues in diabetic rats with no treatment, there was weak insulin-immunoreactivity in a few β-cells in the islet of Langerhans (Fig. 1b).

Exercise training protected the moderate of cells of Langerhans islet in diabetic light-intensity exercise training group, diabetic moderate-intensity exercise training group and diabetic heavy-intensity exercise training group. Nevertheless, in the remaining cells were observed light hydropic degeneration, degranulation and necrosis. In addition, a few cells with picnotic nucleus were indicated. In immunohistochemical staining of the pancreatic tissues in diabetic light-intensity exercise training group there was moderate insulin antigen positivity in the average
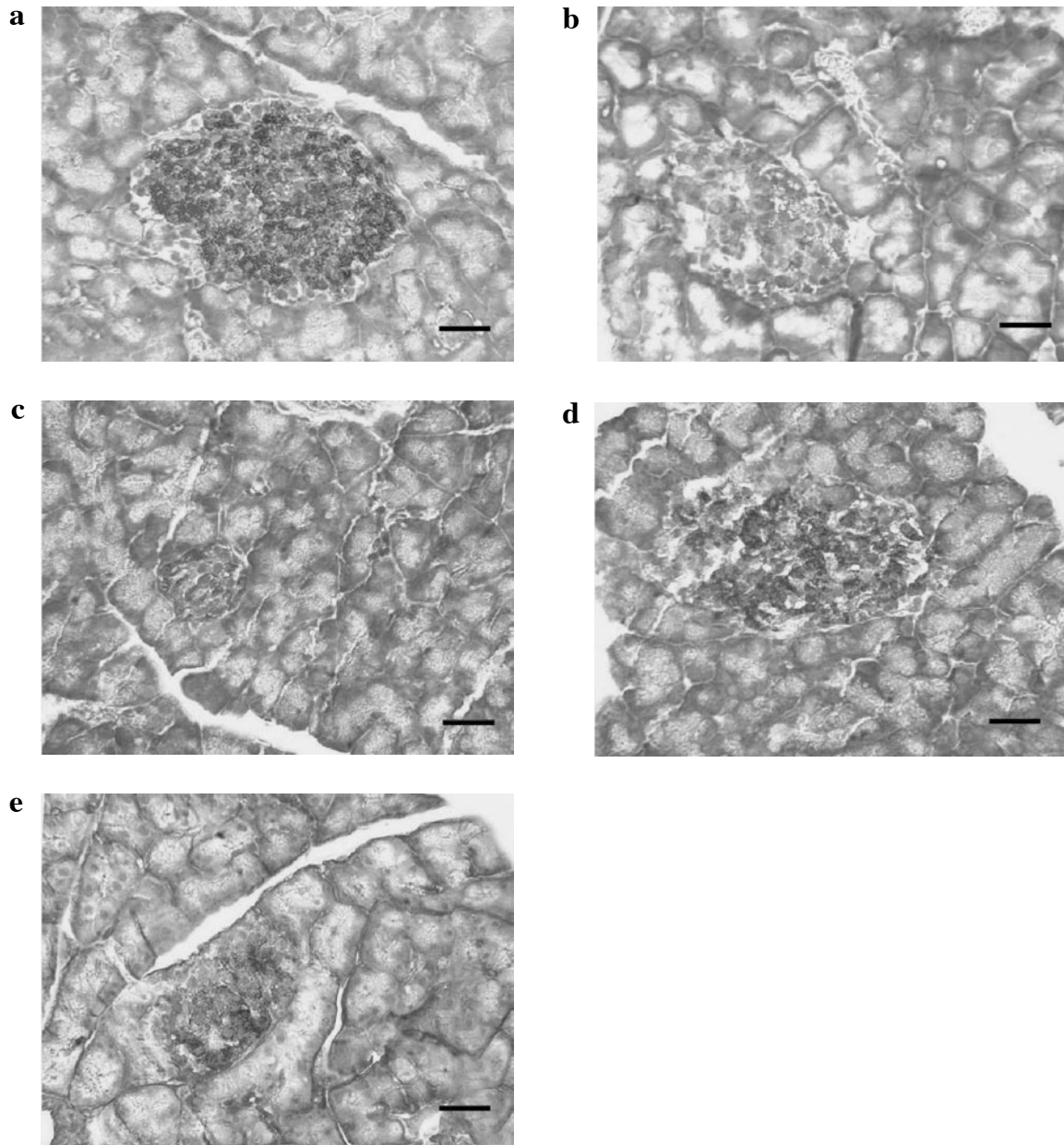
Fig. 1. Immunohistochemical stainings of the pancreatic tissues (bar, 100 μm). (a) Control group: Showing normal cells in the islet of Langerhans and also showing β-cells in the islet of Langerhans that are strong staining with the anti-insulin anti-body. Immunoperoxidase, haematoxylin counterstain. (b) Diabetic group: Shrunken islets of Langerhans displaying degenerative and necrotic changes in diabetic rats with no treatment, and that are weak insulin-immunoreactivity in a few β-cells in the islet of Langerhans. Immunoperoxidase, haematoxylin counterstain. (c) Diabetic light-intensity exercise training group: Exercise has protected the moderate of beta-cells in the islet of Langerhans and shown moderate staining with the anti-insulin antibody. Immunoperoxidase, haematoxylin counterstain. (d) Diabetic moderate-intensity exercise training group: Exercise has protected the majority of beta-cells in the islet of Langerhans and shown moderate staining with the anti-insulin antibody. Immunoperoxidase, haematoxylin counterstain. (e) Diabetic heavy-intensity exercise training group: Exercise has protected the moderate of beta-cells in the islet of Langerhans and shown moderate staining with the anti-insulin antibody. Immunoperoxidase, haematoxylin counterstain.

TABLE 3. *Semiquantitative intensity of staining for insulin and β-cell numbers in pancreatic islets of control, diabetic, diabetic light-intensity exercise training, diabetic moderate-intensity exercise training and diabetic heavy-intensity exercise training groups (n=100 islets)*

Groups	Intensity of staining for insulin and β-cell numbers
Control	+++
Diabetic	±
Diabetic light-intensity exercise training	+
Diabetic moderate-intensity exercise training	++
Diabetic heavy-intensity exercise training	+

−, Absence of β-cells; ±, trace of β-cells, +, ++, +++, relative amounts of β-cells in pancreatic islets (+++ = high, ++ = medium, + = low).

of β-cells of the islets of Langerhans (Fig. 1c). In diabetic moderate-intensity exercise training group, there was moderate insulin antigen positivity in the majority of β-cells of Langerhans (Fig. 1d). In diabetic heavy-intensity exercise training group, there was moderate insulin antigen positivity in the average of β-cells of the islets of Langerhans (Fig. 1e). As shown in Table 3, exercise has a therapeutic and/or protected the intensity of staining for insulin and β-cell numbers significantly in diabetic light-intensity exercise training group, diabetic heavy-intensity exercise training group and especially in diabetic moderate-intensity exercise training group when comparison with untreated diabetic animals ($p<0.05$).

DISCUSSION

The current study examined the effects of swimming exercise on oxidative stress and β-cell damage of STZ-induced diabetic rats. Free radical mechanisms and the possible sources of oxidative stress in the pathogenesis of diabetes and diabetic complications have been extensively studied for years based on in animal models and in patients (Baynes 1991). Diabetics and experimental animal models exhibit high oxidative stress due to persistent and chronic hyperglycemia, thereby deplete the activity of antioxidative defense system and thus promote de novo free radicals generation (Hammers et al. 1991). Oxidative stress has recently been shown

to be responsible, at least in part, for the β-cell dysfunction caused by glucose toxicity. Under hyperglycemia, production of various reducing sugars such as glucose-6-phosphate and fructose increases through glycolysis and the polyol pathway. During this process, ROS are produced and cause tissue damage (Sakurai and Tsuchiya 1988; Hunt et al. 1990; Matsuoka et al. 1997). Exercise training has been known to be effective in type 2 diabetes by increasing insulin sensitivity, there is no knowledge how exercise acts in type 1 experimental diabetes. In our experiment, namely STZ induced experimental type 1 diabetes, we have shown that exercise training is effective not only insulin sensitivity but also β-cell protection. We examined the possible usefulness of the light, moderate and heavy exercises training has a therapeutic preventive and protective effect in diabetes by decreasing oxidative stress and preservation of pancreatic β-cell integrity. Such damaged β-cells often display extensive degranulation when examined histologically, and are clinically associated with the development of diabetes in some model animals for type 2 diabetes (Ihara et al. 1999; Kaneto et al. 1999). Therefore, protection of β-cells against chronic hyperglycemia induced damage is an important target for the treatment of type 2 diabetes. To enable effective prevention (Okamoto and Takasawa 2002) and treatment of glucose toxicity to β-cells, it is essential to understand the biochemical aspects of the phenomenon. To date,

lines of in vitro and in vivo studies have suggested the implication of oxidative stress in the progression of β-cell dysfunction in type 2 diabetes.

Streptozotocin as an antibiotic and anticancer agent has been widely used for inducing diabetes in a variety of animals by affecting degeneration and necrosis of pancreatic β-cells (Uchigata et al. 1982; Merzouk et al. 2000). Although the β-cell cytotoxic action of STZ is not fully understood, it is though to be mediated by the inhibition of free radical scavenger-enzymes thereby enhancing the production of the superoxide radical. The latter has been implicated in lipid oxidation, DNA damage, and sulfhydryl oxidation. In the present study, almost all insulin-positive β-cells were degranulated, degenerated or necrosed in the STZ treated rats leading a decrease in insulin secretion and an increase in blood glucose concentration. STZ causes diabetes mellitus. Diabetes is associated with the generation of ROS causing oxidative damage (Mohamed et al. 1999). Glucose level increased the production of free radicals cell damage markers, such as MDA and conjugated dienes (Cuncio et al. 1995).

Several studies reported that physical exercise induces hypophagia by decreasing energy intake in human (Johnson et al. 1972). For human beings it is very important to stable blood sugar level with regular exercise, health professions, specially nurses who are specialist in diabetes care has to motivate their patients for regular exercise (Ocakcı 1999). It has also been shown that physical exercise enhances insulin sensitivity (Braun et al. 1995; Goodyear and Kahn 1998). Although exercise is known to relieve many of the symptoms of diabetes mellitus, the effect of exercise on appetite control in diabetes is not yet clarified. In our study, the effect of swimming exercise in rats with STZ-induced oxidative stress and β-cell damage was investigated via immuno-histochemistry and biochemistry. Rats are natural swimmers and, thereupon exercise protocols based on swimming are widely used (Bräu et al. 1997). Exercise caused significant decrease in the elevated serum glucose and a slight increase in

the lowered serum insulin concentrations in STZ-induced diabetic rats with the elapse of the experiment. Exercise training protected partly β-cells of Langerhans islet. Nevertheless, in the remaining cells was observed light hydropic degeneration, degranulation and necrosis. The current immunohistochemical examination shows that pancreatic β-cells are destroyed by STZ whereas exercise prevents degeneration of β-cells. Accordingly, interest has recently grown in the role and usage of exercise as a means to prevent oxidative damage in diabetes with oxidative stress (Shima et al. 1993; Gul et al. 2002). There is no more information on the effects of exercise on immunohistochemical examination of diabetic animals in the literature.

This work was conducted to elucidate probable changes in free radicals (lipid peroxides and nitric oxide) and enzymic antioxidants (SOD, CAT and GSH-Px activities). Lipid peroxidation may bring about protein damage and inactivation of membrane bound enzymes either through direct attack by free radicals or through chemical modification by its end products, MDA and 4-hydroxynonenal (Baynes 1991). In our study, erythrocyte and tissue MDA and serum NO concentration was significantly increased in diabetic group with reduction in antioxidant enzyme activities of SOD, GSHPx and CAT. Exercise decreased the elevated MDA and NO and also increased the reduced antioxidant enzyme activities. Our result is consistent with the other studies results (Favier et al. 1987; Shima et al. 1993; Braun et al. 1995; Nikolovski et al. 1996; Bräu et al. 1997; Goodyear and Kahn 1998; Derouich and Boutayeb 2002; Gul et al. 2002; Henriksen 2002), who indicated an increase in lipid peroxides and a decrease in antioxidant enzymes in diabetes mellitus.

These findings suggest that exercise training has therapeutic preventive and protective effects in diabetes by decreasing of oxidative stress and preservation of pancreatic β-cell integrity.

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