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The Role of Metformin and Aerobic Exercise on The Hepatic Ischemia-Reperfusion Injury in Streptozotocin-Induced Diabetic Mice

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ABSTRACT: Due to prolonged hyperglycemia many of the diabetic patients suffer from complications such as liver damage. Diabetic patients are known to have the need for liver surgeries more than non-diabetic do. The ischemia reperfusion injuries (IRI) are one of the main complications of these surgeries and the IRI-related increase in oxidative stress has been known to be higher in diabetic patients. Metformin and aerobic exercise are important tools being used especially in type-2 diabetes. However, their effects and roles in liver IRI in type-1 diabetic patients are not known. This study aimed to investigate the effects of metformin and exercise on hepatic IRI in diabetes in streptozotocin induced type-1 diabetic mice. Diabetes was induced by streptozotocin and two weeks after the disease developed, mice were started to treat with metformin and/or aerobic exercise during four-weeks. Blood glucose levels of the mice were measured again and the glucose tolerance test (OGTT) was performed for each mouse. The day after OGTT, ischemia was performed for 45 minutes in the liver and then reperfusion was provided for 5 hours. The liver of the mice was isolated at the end of the experiments. The malondialdehyde, superoxide dismutase and nitrite levels were measured with colorimetric analysis. Metformin reduced the insulin resistance alone and together with aerobic exercise. Oxidative stress in the liver after IRI was diminished both with metformin and/or aerobic exercise in diabetic mice. Our study indicated that metformin accompanied with aerobic exercise might be an important treatment strategy for preventing the IRI in the liver of type-1 diabetic patients.

KEYWORDS: Liver injury; ischemia-reperfusion; metformin; aerobic exercise; type-1 diabetes.

1. INTRODUCTION

Diabetes mellitus (DM) is one of the most common chronic diseases in worldwide. The patients with DM also suffer from serious complications related with the disease. Hyperglycemia, the main symptom of DM, causes acute and chronic complications on the body such as ketoacidosis, retinopathy, nephropathy, foot and skin problems, neuropathy and cardiovascular damages. The ischemia-reperfusion (IR) damage in vital organs is higher in patients with diabetes than in healthy individuals [1]. In the liver one of the primary concerns of liver transplantation is IR injury (IRI). Liver is one of the most affected organs by hyperglycemia. A previous study showed that in STZ-induced diabetic mice and rats, DM exacerbated liver IRI mostly via oxidative stress and inflammation pathways [2].

In diabetic patients, the regular aerobic exercise reduced the oxidative stress and found beneficial in controlling glycemia [1, 3]. Aerobic exercises also diminished liver injury reducing oxidative stress in rats and the pre-operative exercise alleviated the IRI depending on a link between hepatic immune modulation and oxidative stress in mice [4, 5]. Metformin is an important antidiabetic that is used to control hyperglycemia in type-2 diabetes. However, in recent years the effects of metformin in type-1 diabetes have also been taken attention. There have been studies showing that metformin treatment may be beneficial reducing the frequency of insulin usage and delaying the diabetes-related complications in patients with type 1 diabetes [6, 7]. Although it is a common idea that there is no insulin resistance in type-1 diabetes; the term "double diabetes" was used for the patients that have significant insulin resistance beside their type-1 diabetes [8]. This situation is described as an independent risk factor for vascular injuries [9]. Many recent studies indicated the increase in body mass index (BMI) and high dose insulin requirements in type-1 diabetic patients and it is known that metformin usage reduced the BMI [10, 11]. Furthermore, it has been shown that metformin

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increases the antioxidant capacity of the body [12, 13]. In liver, the metformin pretreatment had been found very effective in reducing hepatic IRI in the patients have fatty liver and healthy subjects by attenuating oxidative species formation or inflammation [14, 15]. However, the studies investigating the role of metformin in type-1 diabetes and diabetes-related complications are limited. There is still no consensus about this issue and metformin still not approved by FDA in type-1 diabetes [16].

Both aerobic exercise and metformin has been found effective in liver IRI via diminishing the oxidative stress. They are also effective in controlling blood glucose levels and reducing insulin resistance. However, there is no study investigating the role of metformin or synergistic effect of it with aerobic exercise in IRI in liver and the effects on diabetic complications were not shown. We hypothesized that metformin treatment and aerobic exercise can be effective in liver IRI in diabetic condition depending on their antioxidant effects while regulating blood glucose. Considering the inadequacy of studies investigating the effect of metformin on type 1 diabetes; we also aimed to evaluate and compare the effects of metformin and regular exercise on type-1 diabetic mice.

2. RESULTS

2.1. Development of Type-1 DM and Effects of Aerobic Exercise and Metformin Treatment on Blood Glucose

Two weeks after the STZ injection blood glucose levels were significantly increased compared with control group and type-1 DM was developed in mice (*$p<0.05$ vs two weeks after vehicle injected control group; Figure 1).

After four-week treatment, the blood glucose level of the vehicle-treated STZ group was still significantly high compared with vehicle treated-control group (θ $p<0.05$ vs four weeks vehicle treated control group; Figure 1). The four-week aerobic exercise slightly decreased the blood glucose level in the diabetic mice; however, this reduction was not reached statistical significance. On the other hand, metformin treatment alone and aerobic exercise beside metformin treatment during four weeks provided significant reduction in blood glucose in the type-1 diabetic mice compared to four-week vehicle-treated STZ group (#$p<0.05$ compared to four-week vehicle treated STZ group; Figure 1).

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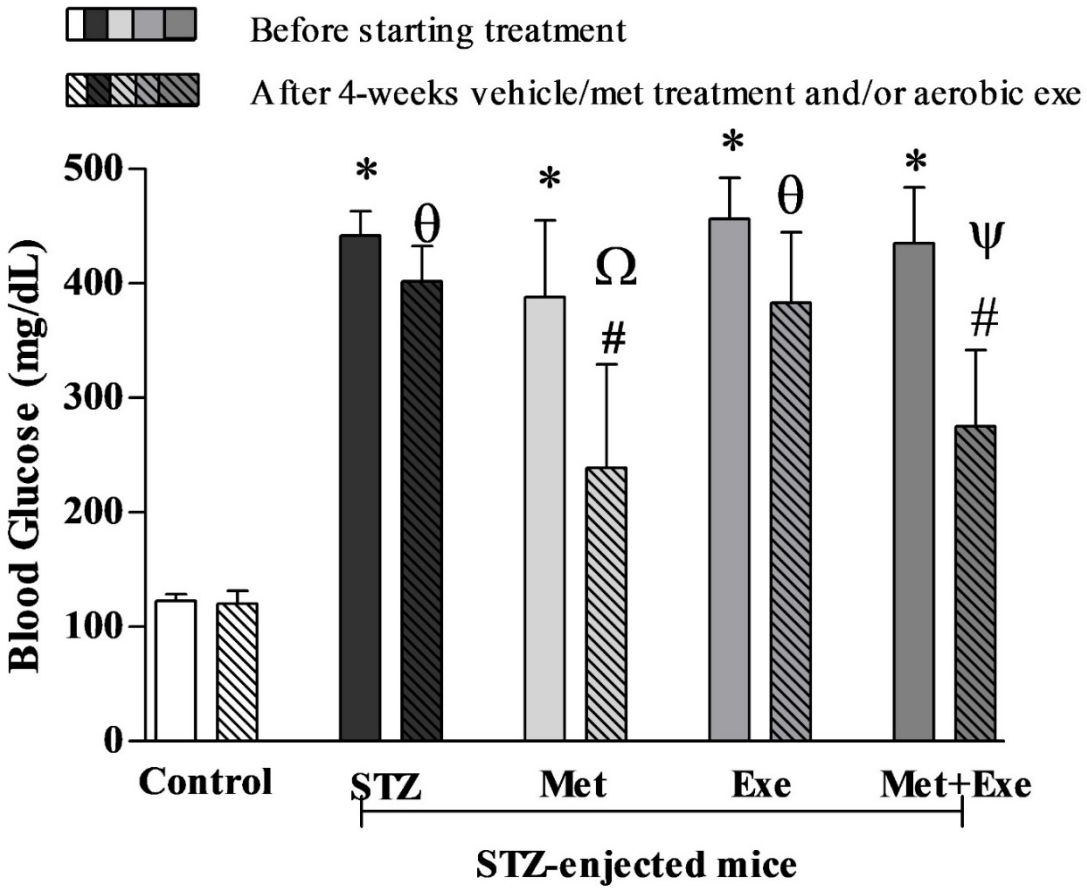

Figure 1. The plasma blood glucose levels, 2-weeks after STZ injection-just before starting treatment protocols and the plasma blood glucose levels after 4-weeks vehicle/metformin treatment and/or aerobic exercise. Data were presented as mean±SEM. The statistical analysis was performed with one-way analysis of variance (ANOVA) post-hoc Tukey test for comparing the five different groups and inside each group paired Student's t test for assessing the changes the blood glucose after 4-weeks treatment protocols. *p<0.05 control vs other groups before starting treatment-2 week after STZ injection; #p<0.05 vs. four weeks' vehicle treated STZ group. θ p<0.05 control administrated vehicle for 4-weeks vs other groups that were treated for 4 weeks. Ω p<0.05 before treatment vs after Met. Ψ p<0.05 before treatment vs after Met+Exe.

Met: Metformin, STZ: streptozotocin, Exe: exercise.

Metformin treatment were also significantly reduced the blood glucose levels after four weeks compared the blood glucose levels of the same mice before starting experiments (Ω p<0.05 vs before starting metformin treatment). The aerobic exercise accompanying metformin treatment also provided reduction in blood glucose comparing to the blood glucose levels of mice before starting exercise and treatment (Ψ p<0.05 vs before four-week treatment protocol).

2.2 Effects of Aerobic Exercise and Metformin Treatment on Insulin Sensitivity in Type-1 Diabetic Mice

The insulin resistance of the mice was assessed with OGTT. The insulin resistance was observed in the STZ group compared to control group (*p<0.05; Figure 2). Metformin treatment alone and together with exercise increased the insulin sensitivity compared with vehicle-treated STZ group (#p<0.05, Figure 2). Although a tendency to decrease was observed in the insulin resistance of the exercise group it did not reach statistical significance (Figure 2).

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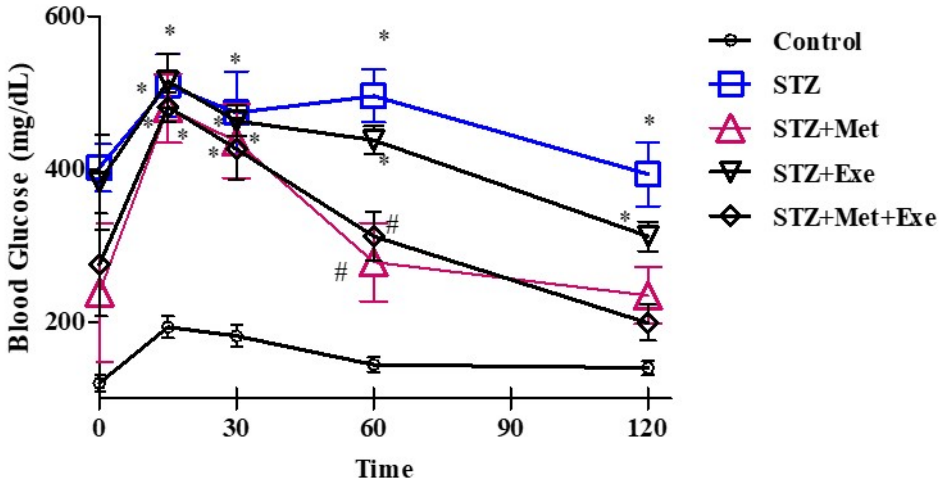

Figure 2. The OGTT of mice with STZ induced type-1 DM and the effect of metformin and/or aerobic exercise in the insulin resistance. Data were presented as mean±SEM. The statistical analysis was performed with one-way ANOVA post-hoc Tukey test. *p<0.05 vs. control and #p<0.05 vs. STZ groups (n=6 for each group). Met: Metformin, STZ: streptozotocin, Exe: exercise.

2.3. Biochemical Analysis

2.3.1. MDA levels

MDA levels in the liver tissues were higher in diabetic mice than control mice after IRI (*p<0.05, Figure 3). In diabetic mice performing exercise before the IRI, the MDA levels were found significantly lower compared to STZ induced diabetic group (#p<0.05, Figure 3). Furthermore, the metformin treatment alone or in combination with exercise the MDA level is as similar as the level of control mice (#p<0.05, Figure 3).

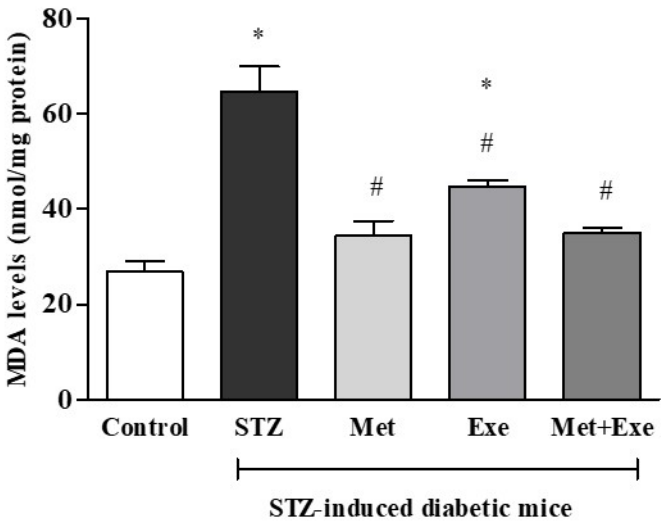

Figure 3. The MDA levels of the liver tissues of the all groups after ischemia reperfusion injury. Data were presented as mean±SEM. The statistical analysis was performed with one-way ANOVA post-hoc Tukey test. *p<0.05 indicates statistical significance compared to control mice, #p<0.05 compared to SZT-induced diabetic mice (n=6 for each group). Met: Metformin, STZ: streptozotocin, Exe: exercise.

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2.3.2. SOD measurement

The superoxide dismutase (SOD) levels in liver tissues were lower in STZ group compare to control mice after IRI (*p<0.05, Figure 4). The treatment of diabetic mice with metformin before IRI prevented the SOD reduction, and this prevention was enhanced by concomitant aerobic exercise (#p<0.05; Figure 4). In the group that only performed aerobic exercise in diabetic mice during 4 weeks; the SOD levels were higher compare to diabetic group. However, there were no statistical significance (Figure 4).

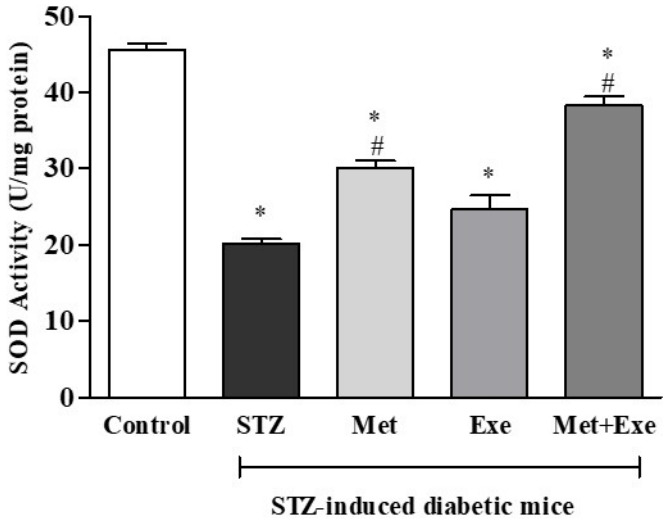

Figure 4. The liver tissue SOD levels control and diabetic mice. The SOD activity reduced in STZ-induced diabetic mice and both metformin treatment and metformin treatment+aerobic exercise improved SOD levels compared to STZ group. Data were presented as mean±SEM. The statistical analysis was performed with one-way ANOVA post-hoc Tukey test. *p<0.05 indicates statistical significance compared to control mice, #p<0.05 compared to STZ-induced diabetic mice (n=6 for each group). Met: Metformin, STZ: streptozotocin, Exe: exercise

2.3.3. Nitrite measurement

The nitrite levels in the livers of STZ-induced diabetic mice were significantly lower compared with control (*p<0.05, Figure 5). The metformin treatment and aerobic exercise slightly increased the nitrite levels. On the other side the increase in the group performing aerobic exercise concurrently with metformin treatment was found statistically significant compared to STZ group (#p<0.05, Figure 5).

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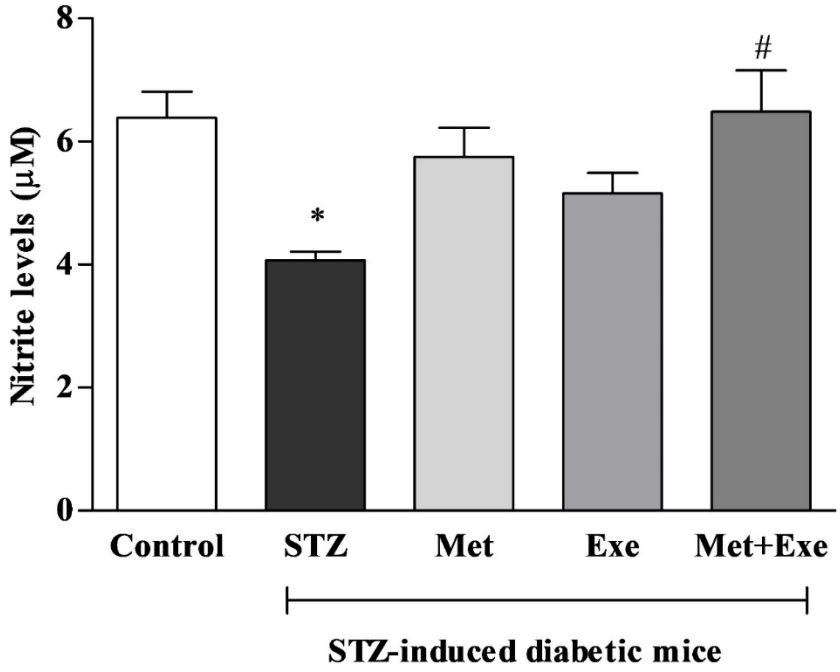

Figure 5. The liver tissue nitrite levels control and diabetic mice. The nitrite levels reduced in STZ-induced diabetic mice and metformin treatment+aerobic exercise ameliorated nitrite levels compared to STZ group. Data were presented as mean±SEM. The statistical analysis was performed with one-way ANOVA post-hoc Tukey test. *p<0.05 indicates statistical significance compared to control mice, #p<0.05 compared to STZ-induced diabetic mice (n=6 for each group). Met: Metformin, STZ: streptozotocin, Exe: exercise

3. DISCUSSION

Increasing insulin sensitivity is one of the main purposes of type-2 diabetes. However, in recent years, it has been discussed that increasing insulin sensitivity in type 1 diabetes may be an important part of the treatment and can be effective for preventing the diabetes-related complications [16, 17, 18]. American Diabetes Association underlines that due to insulin resistance type-1 diabetes patients may need higher doses of insulin and diabetic complications may increase [19]. Therefore, the adjunctive addition of metformin to the treatment for reducing insulin-usage in type-1 diabetes has gained importance. Our study supported this viewpoint with providing a significant reduction in the blood glucose levels in STZ-induced diabetic mice after 4-weeks metformin treatment. The chronic usage of metformin also increased the insulin sensitivity as we demonstrated with OGTT. Furthermore, the beneficial effects of aerobic exercise in insulin resistance both in type-1 and type-2 diabetes have been demonstrated in many studies and it has been shown that exercise stimulates glucose uptake of insulin and reduces HbA1c [20, 21]. Therefore, the regular, moderate-intensity aerobic exercise are recommended for diabetic patients in many years [22]. In our study, insulin resistance and blood glucose levels of mice receiving metformin treatment along with exercise were significantly reduced. On the other hand, the blood glucose levels of mice that performed only aerobic exercise by swimming for 4 weeks were slightly reduced. In our study, four-week aerobic exercise did not provide an extra efficiency of blood glucose levels comparing with only metformin treatment. It is known that long-term exercise may be better in controlling blood glucose [21]. The aerobic forms of exercise were recommended to perform for more than 3 months for a chronic glycemic control [23]. Thus 4-weeks aerobic exercise in our study may not have been enough for reducing the blood glucose levels significantly.

Liver is one of the most affected organs by diabetes in the body [2, 24]. It has been reported that more than 25 % of the liver transplant patients have pre-existing diabetes and the transplantation can be unsuccessful very commonly in the type-1 diabetic patients [25]. Therefore, regulating the blood glucose and reducing diabetes-related oxidative stress prior to surgical procedure is crucial for diminishing the IRI [26, 27]. In diabetic transgenic mice that overproduced SOD, the diabetic complications like nephropathy, retinopathy and cardiomyopathy were less common [28]. Our study is consistent with other investigations showing that oxidative stress in IRI is higher in STZ-induced diabetic mice compared to control animals [2]. Regular aerobic exercise alleviates the IRI via reducing oxidative stress parameters in healthy people. The antioxidant defense

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mechanism was strengthened in the diabetic patients that performed regular aerobic exercise. [3, 29]. Metformin also diminished the oxidative stress in diabetes and alleviated the IRI in the many different tissues and organs of normoglycemic conditions [30]. However, as far as we know, for the first time we demonstrated that both regular aerobic exercise and metformin provided beneficial effects on liver IRI injury dependent changes of oxidative stress markers in mice. Both of them reduced MDA levels while increasing SOD and nitrite levels in the liver of STZ induced diabetic mice. The results of this study indicated that metformin treatment together with regular aerobic exercise may provide significant support reducing the oxidative stress and hepatic IRI in type 1 diabetes.

On the other side our study has some limitations. Although, STZ-induced diabetes model in mice is one of the most used rodent models in diabetes research it is known that the model does not exactly mimic the disease. Therefore, it has limitations to interpreted the results to human. Since STZ is a toxin that can be caused releasing of free nitric oxide and this lead to produce free oxygen radicals.[31]. Furthermore, it is known that mice have different antioxidant system than humans and this could be affecting the results of oxidative stress parameters [32]. Another important point is because of the interruption of the most beta cells, STZ induced diabetic mice model not always provided insulin resistance. However, we selected the insulin resistant mice performing OGTT and aimed to assess the type-1 diabetes with insulin resistance. Consequently, in our study the recurrent hyperglycemia was observed in the mice that lead to insulin resistance and biochemical changes in the body.

4. CONCLUSION

Exercise and metformin reduced the insulin resistance that can also be seen in type-1 diabetes. The fact that the better oxidative stress levels in IRI-damaged livers of mice treated with both metformin and exercise compared to other groups, demonstrating the preventative role of them in not only type-2 but also type-1 diabetes.

5. MATERIALS AND METHODS

5.1. Animals and Experimental Design

Adult, 25-30 g weight Balb/C male mice were used throughout the experiments. Balb/C mice were frequently used in both exercise and diabetes studies [33, 34]. Animals were acclimatized for a week before starting the experiments and were kept in a room at 22 ±2°C temperature with 12h day/12h night circle. They had unlimited access to food and water during all process. All animal experiments were performed with the approval of Hacettepe University Animal Experimentations Local Ethics Board (2021-03/04).

All animals except controls were injected with STZ for developing type -1 diabetes. The control animals (n=6) were injected with only the vehicle of STZ. Two weeks after the injections the development of the hyperglycemia was confirmed with measuring the blood glucose levels. The STZ injected animals were divided into four with six animals in each group. Totally, there were five groups as following: the control, the diabetes, the diabetes treated with only metformin, the diabetes only performing aerobic exercise and the diabetes performing aerobic exercise and treated at the same time with metformin groups. There were six animals in each group and except control group all of the animals have hyperglycemia before starting the metformin and exercise. The metformin treatment and aerobic exercise were applied during four weeks to the type-1 diabetic mice.

5.2. Streptozotocin Induced Type-1 Diabetes Model

Type-1 diabetes was inducted with intraperitoneal (i.p.) injection of freshly prepared high dose single STZ (Sigma Aldrich, Germany) dissolved in 0.5 M citrate buffer at pH 4.5 at 200 mg/kg/0.2 mL doses [35]. The animals in the control group were inject ed with i.p. 0.2 ml citrate buffer. The mice were fasted for 4 hours prior to injection. After STZ injection, mice were taken 10% sucrose solution as drinking water for 24 hours to prevent sudden death caused by destruction of insulin. Two weeks after the injection, the tails of the mice were cut and their blood glucose were measured with a glucometer (FreeStyle Libre, Abbott®). The mice that have blood glucose above 200 mg/dl were accepted diabetic. The mice that have more than 550 mg/dl blood glucose level were not accepted experiments to be able to observe the insulin resistance.

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5.3. Mice Aerobic Exercise and Metformin Treatment

Two weeks after the STZ injection and confirmation the diabetes with measurement of blood glucose; the aerobic exercise and/or metformin administration were started in the related groups. The weight-bearing swimming was used as an aerobic exercise model in mice. Briefly, the mice were floated in glass containers 19 cm high and 12 cm in diameter in water at 32±3 °C for totally four weeks. Experiments were performed 5 days a week, at the same hours and light phase of the day. Swimming time was kept for 10 minutes in the first week, and this time will be increased to 1 hour in the remaining 3 weeks. A metal weight of 2% of their body weight was attached to the animals after the preliminary experiments with different metal weights [36]. The metformin was administered orally with gavage at 250 mg/kg doses [37] every day at the same hours, 2 hours after the completing aerobic exercise period during these four weeks.

5.4. Oral Glucose Tolerance Test

OGTT was performed to assess the insulin sensitivity and the effect of aerobic exercise and metformin on it. OGTT was performed in the light phase of the day, starting a day after the end of the 4-week metformin treatment and/or exercise protocols. Mice were fasted for 6 hours before the test; the blood glucose was measured first and then 2g/kg glucose was administrated orally for each of the mice. The blood glucose levels were measured in 15th, 30th, 60th and 120th minutes after glucose administration.

5.5. Liver Ischemia/Reperfusion Model in Mice

Liver IRI was performed in the light phase of the day starting two hours after the OGTT was completed. Liver IRI was inducted under ketamine/xylazine anesthesia (90 mg/kg-10 mg/kg). The ischemia was performed by an atraumatic clamp just above the branch to the right lateral lobe, covering the portal vein, hepatic artery, and bile duct. The clamp was removed after 45 minutes of ischemia and the abdomen was closed with a 4.0 silk suture. The mice will be euthanized by cervical dislocation 5 hours after awaken from anesthesia. The ischemia-induced liver lobes were isolated, placed into liquid nitrogen and were preserved in -80°C before the biochemical analysis.

5.6. Biochemical Analysis

Oxidative stress in the liver tissues was assessed with measuring the MDA, SOD and nitrite levels. The liver pieces were cut at the same weight were homogenized with different homogenization buffers (1:10g/mL). Liver tissues were homogenized using ultrasonic homogenizer (Bandelin, Germany) with phosphate buffer saline for SOD and nitrite analysis and then with 1.15% potassium chloride solution for MDA analysis and centrifuged at 14 000 g for 20 minutes. The supernatants were collected and preserved in -80°C until performing the experiments.

5.6.1. MDA measurements

The MDA levels were measured according to thiobarbituric acid (TBA) reaction method [38]. The test was performed according to Lipid Peroxidation (MDA) Assay Kit (Sigma-Aldrich, Germany). This procedure is depending on the measurement of the colorimetric product of the reaction of MDA with TBA at 532 nm. A standard curve was used for measuring the MDA levels of the samples.

5.6.2. SOD measurements

The SOD levels of the liver tissues were measured according to the manual of the SOD Assay Kit (Invitrogen, United States of America). Briefly, the activity of SOD was determined with the decrease of superoxide anions that interact with the water-soluble tetrazolium salt (WST) dye, yielding color at 450 nm. The decrease in the color signal indicates SOD inhibition [39]. One unit of SOD is the amount of enzyme causing half the maximum inhibition of 1.5 mM nitro blue tetrazolium reduction in the presence of riboflavin at pH 7.8 and 25°C. The SOD levels were analyzed using the obtained standard curve.

5.6.3. Nitrite measurements

The determination of nitrite in homogenates of liver tissues was performed using the Griess reaction that was based on the conversion of nitrite to a purple-colored azo-dye [40]. Briefly, 100 µl of supernatant was transferred to the wells of the 96-well microplate. 100 µl of Griess reagent was added and it was kept at room temperature for 10 minutes. The absorbance was be measured a wavelength of 540 nm. The nitrite levels were analyzed using the obtained standard curve and results are presented as a ratio to ml supernatant.

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5.7. Statistical Analysis

One-way analysis of variance (ANOVA) post hoc Tukey test was performed for comparing the groups, Student's t test was used for paired analysis. The results were presented as mean ±standard error mean (SEM). Graphpad Prism vs 5.0 was used for analysis (California, USA).

Author contributions: Concept – G.T., O.T.A.; Design – G.T., O.T.A.; Supervision –O.T.A.; Resources – G.T.; Materials – G.T.; Data Collection and/or Processing – G.T.; Analysis and/or Interpretation – G.T..; Literature Search – G.T., O.T.A.; Writing –G.T.; Critical Reviews – O.T.A.

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