

## Original Article

## Pre- and Post-Treatment of Streptozocin Administered Rats with Melatonin: Effects on Some Hepatic Enzymes of Carbohydrate Metabolism

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**Background:** Melatonin, found in high concentrations in the pineal gland, organs within the digestive system and in some plants and fungi, acts as an antioxidant which decreases reactive oxygen species in streptozocin-induced diabetic rats, raises insulin secretion by the pancreatic  $\beta$ -cells and increases the number of insulin receptors on hepatocyte membranes.

**Materials and Methods:** The protective and therapeutic effects of melatonin feeding in streptozocin-induced diabetic rats were studied. Streptozocin administered rats were gavaged with melatonin, pre- and post-treatment, at a level of 5 mg/kg body weight daily for a period of 15 days. Levels of plasma glucose, cholesterol, triacylglycerol, oral glucose tolerance test, and some hepatic enzymes of carbohydrate metabolism including insulin inducible glucokinase, hexokinase and glucose 6-P dehydrogenase were measured using standard methods and compared with the values in normoglycemic and diabetic control groups.

**Results:** Both pre- and post-treatment of the streptozocin administered rats with melatonin normalized plasma glucose, cholesterol, and triacylglycerol, improved oral glucose tolerance test and increased hepatic glucokinase, hexokinase and glucose 6-P dehydrogenase specific activities to the levels seen in normal rats.

**Conclusion:** Melatonin pre-treatment prevents the injurious effects of streptozocin in rats. In streptozocin induced diabetic animals, post-treatment with this antioxidant normalizes both blood and liver constituents which were ameliorated by streptozocin.

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### Introduction

**D**estruction of pancreatic  $\beta$ -cells by streptozocin produces diabetes in experimental animals.<sup>1-3</sup> Formation of reactive oxygen species (ROS) is thought to be a mediator of the cytotoxic actions of streptozocin.<sup>3</sup> Organisms have developed several defense mechanisms to protect their cells against ROS. Such mechanisms include use of antioxidant enzymes and small antioxidant molecules such as vitamins C, E and flavonoids. Antioxidant enzymes

metabolize ROS into non-toxic products as the first line of defense against toxic free radicals. Injection of superoxide dismutase or treatment with vitamins C or E diminishes oxidative stress in diabetic rats.<sup>4-6</sup>

Melatonin (N-acetyl-5 methoxy tryptamine), an endogenous neurohormone produced by the pineal gland, also decreases ROS in streptozocin-induced diabetes.<sup>7-9</sup> Melatonin exerts its antioxidant effects by either simple diffusion into cells or via G-protein dependent receptors through the induction of antioxidant enzyme synthesis.<sup>10</sup> In this way, melatonin neutralizes the effects of free radicals.<sup>11</sup> Melatonin may also react directly with free radicals and produce the melatonyl radical which is capable of neutralizing superoxide anions.<sup>12</sup> In addition to the pineal gland, other tissues as well as bacteria, fungi, protozoa, plants and invertebrates can synthesize melatonin from the amino acid,

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tryptophan.<sup>13</sup>

It has been demonstrated that intraperitoneal injection of 200 µg melatonin per kg body mass daily for 15 days increases the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in streptozocin-induced diabetic rats.<sup>14</sup> Melatonin also increases the activities of glucose 6-P dehydrogenase (G6PD) in the livers and brains of diabetic animals resulting in an increase in the level of NADPH required for glutathione reductase activity.<sup>15</sup> In addition, melatonin prevents hyperglycemia in streptozocin-induced diabetic rats through inhibition of nitric oxide synthase (NOS).<sup>16</sup>

Removal of the pineal gland in rats results in decreased insulin secretion by pancreatic β-cells and induces hyperglycemia, together with a decrease in insulin receptors and an increase in glucagon receptors on the surfaces of hepatocytes. Administration of melatonin reverses such effects.<sup>17,18</sup> It has been demonstrated that melatonin attenuates plasma cholesterol levels in hypercholesterolemic mice.<sup>19</sup> Daily melatonin administration at 100 and 200 µg per kg body mass was found to normalize blood glucose levels of streptozocin diabetic rats and significantly reduce plasma triacylglycerol (TG) and cholesterol levels.<sup>20</sup>

Although the effects of melatonin on insulin secretion and the reduction of plasma glucose levels are well established, the role of this compound in the metabolism of glucose is not well understood. Therefore, in this investigation, both the protective effects of an intragastric administration of melatonin in preventing streptozocin-induced diabetes (pre-treatment with melatonin) and its therapeutic effects (post-treatment with melatonin) in abolishing streptozocin-induced diabetes were investigated. The effects of both treatments on blood glucose levels, oral glucose tolerance test (OGTT), activities of liver glucokinase, hexokinase and G6PD enzymes, as well as plasma TG and cholesterol levels were studied in streptozocin administered rats and compared with their respective parameters in normoglycemic animals.

## Materials and Methods

### Reagents

Fatty acid free bovine serum albumin and *Leuconostoc mesenteroides* G6PD were acquired from Roche Chemical Company (Mannheim,

Germany). Na<sub>2</sub>-ATP, Na<sub>2</sub>-NADP<sup>+</sup>, Na<sub>2</sub>-NAD<sup>+</sup> and Na<sub>2</sub>-glucose 6-P were purchased from Fluka Chemical Company (Buchs, Switzerland). Bovine serum albumin, 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer, and melatonin were acquired from Sigma Chemical Company (St. Louis, MO, USA). Streptozocin vials containing 1 g streptozocin and 220 mg citric acid were secured from the Upjohn Company (Kalamazoo, MI, USA). Streptozocin was reconstituted immediately before use with 9.5 ml of 0.9% NaCl to a final pH of 3.5 – 4.5 according to the manufacturer's instructions.

Enzymatic kits used for the determination of glucose, cholesterol and TG were purchased from Pars Azmoon Company (Tehran, Iran). All other reagents were obtained through other commercial sources.

### Animal experiments

Twelve groups of adult male Sprague Dawley derived rats born and raised in Shiraz University of Medical Sciences' Animal Quarters (8 rats per group) weighing 250 – 270 g were used. The animals were kept in a temperature controlled environment (22 –25°C) with a 12 h light/dark alternating cycle.

Animals were fed rat chow (Pars Dam, Tehran, Iran) and water ad libitum. The experimental protocol was approved by the Ethics Committee of our University.

Out of the 12 groups of rats, 6 were used for melatonin pre-treatment and 6 for melatonin post-treatment with streptozocin. Among the 6 pre-treatment groups, groups 1 and 2 were used as healthy controls and were gavaged with 1 mL of a 1:500 (v/v) ethanol:water daily for 15 days. Groups 3 and 4 were also treated for 15 days as in groups 1 and 2, but at the end of this period 0.5 mL of a 20 mg/mL streptozocin (40 mg/kg body mass) was injected into each animal through their caudal veins, while the control animals (groups 1 and 2) received 0.5 ml of 0.9% NaCl by the same route. Groups 5 and 6 of the pre-treatment animals received, by gavage, 1 mL of melatonin (1.25 mg/mL) which amounted to an average of 5 mg/kg body mass<sup>20</sup> per day for 15 days prior to administration of the intravenous (iv) dose of streptozocin as in groups 3 and 4. Serum glucose was measured one week from the time of streptozocin administration.

In the post-treatment experiments, groups 7 and 8 were healthy control groups which received

0.5 mL of 0.9% saline via the caudal vein and after one week were gavaged with a 1 mL dose of 1:500 (v/v) ethanol:water for a period of 15 days. Groups 9, 10, 11, and 12 (whose numbers were originally greater than 10) were made diabetic at the beginning of the experiment by the same iv dose of streptozocin as in groups 3 and 4. After one week from the time of streptozocin administration, serum glucose was measured and 8 animals in each group with blood glucose levels greater than 17.0 mM were chosen as the diabetic experimental animals. After this period, diabetic animals in groups 9 and 10 received, by gavage, 1 mL of 1:500 (v/v) ethanol:water for 15 days. Those in groups 11 and 12 were treated by gavage with the same dose of melatonin for 15 days as groups 5 and 6 of the pre-treatment protocol. Administration of all chemicals started at 9:00 a.m. daily. At the end of the experimental period, pre-treatment groups 1, 3, and 5 in addition to groups 7, 9, and 11 from the post-treatment experiments were sacrificed in the fed state and their livers were removed and used to assay for glucokinase, hexokinase and G6PD. Groups 2, 4, and 6 of the pre-treatment as well as 8, 10 and 12 of the post-treatment experiments fasted for 24 hours. Subsequently, blood was removed from their tails by hematocrit tubes and used for serum glucose, cholesterol and TG measurements as well as OGTT.

#### Preparation of liver homogenates

In order to measure G6PD, liver samples from the fed animals were washed and homogenized with cold 0.154 M KCl at a volume of 4× their respective weights as described by Vessal et al.<sup>21</sup> and the supernatants obtained after centrifugation at 27,000×g for 25 minutes at 4°C were used for G6PD assay.

In order to assay glucokinase and total hexokinase activities, portions of the livers from fed animals were washed twice with a homogenization buffer (pH 7.4) containing Na-HEPES, 50 mM; KCl, 100 mM; EDTA, 1 mM; MgCl<sub>2</sub>, 5 mM; and dithiothreitol, 2.5 mM. The washed liver samples were stored at -70°C until analyzed.

#### Enzyme assays

Hepatic G6PD was assayed spectrophotometrically using glucose 6-P and NADP<sup>+</sup> as substrates and liver supernatant as the enzyme source. The increase in absorbance at 340

nm due to the formation of NADPH was recorded and enzymatic activity was measured according to the method of Bottomley et al.<sup>22</sup>, as described by Vessal et al.<sup>21</sup> Enzyme activities were expressed in milliunit (mU) per mg protein. One mU of the enzyme corresponded to the amount of enzyme which produced 1 nmol NADPH per minute under assay conditions at 25°C.

To determine total hexokinase and glucokinase, 1 g of the frozen liver tissue samples were homogenized in 9 mL of HEPES homogenization buffer (pH 7.4) as described by Vessal et al.<sup>23</sup> The supernatant obtained upon centrifugation of the homogenate at 12,000 g for 1 hour at 4°C was used in the coupled enzyme assay of Davidson and Arion<sup>24</sup>, and Ferre et al.<sup>25</sup> as described by Vessal et al.<sup>23,26</sup> In this assay, the conversion of two different concentrations of glucose to glucose 6-P in the presence of ATP coupled with the oxidation of glucose 6-P by *Leuconostoc mesenteroides* G6PD in the presence of NAD<sup>+</sup> were used for the assay of glucokinase and total hexokinase. The increase in absorbance at 340 nm due to the formation of NADH was recorded. Hexokinase and glucokinase activities were expressed in mU/mg protein, where 1 mU was the amount of the enzyme that produced 1 nmol of NADH per minute under assay conditions at 25°C.

Protein in the liver supernatants was measured with the biuret reagent.<sup>27</sup>

#### Oral glucose tolerance test (OGTT)

OGTT in the groups which fasted for 24 hours was performed by the procedure of Young et al.<sup>28</sup> as previously described.<sup>23,26</sup> Such groups included healthy animals, streptozocin administered animals and streptozocin + melatonin administered animals in both pre- and post-treatment groups. At the end of the respective experimental periods, the serum glucose levels of the streptozocin+melatonin groups approached the normal group. Then, all animals were given water but no food for 24 hours before the OGTT was performed. Blood glucose levels were measured after the fasting period and were considered as 0 time blood glucose concentration. Each animal was then gavaged with a 1 mL glucose solution which contained 0.78 g glucose (3 g per kg body mass). Blood glucose concentrations were measured at 45, 90 and 135 minutes after feeding (see results).

#### Measurement of plasma glucose, cholesterol and TG

Plasma glucose, cholesterol, and TG were

**Table 1.** Effects of melatonin pre-treatment on fasting blood glucose, cholesterol, triacylglycerol (TG), hepatic hexokinase, glucokinase, and G6PD activities in streptozocin injected rats

Experimental groups <sup>1</sup>	Fasting plasma glucose (mM)	Plasma cholesterol (mg/dL)	Plasma TG (mg/dL)	Hexokinase (mu/mg protein)	Glucokinase (mu/mg protein)	G6PD (mu/mg protein)
Normoglycemic control	6.7±0.2 <sup>2a</sup>	53.7±3.9 <sup>a</sup>	48.0±3.2 <sup>a</sup>	0.96±0.06 <sup>a</sup>	1.4±0.32 <sup>a</sup>	9.9±0.5 <sup>a</sup>
Normoglycemic +melatonin	ND	ND	ND	0.97±0.08 <sup>a</sup>	0.64±0.07 <sup>c</sup>	9.4±0.9 <sup>a</sup>
Diabetic control	19.1±1.3 <sup>b</sup>	124.9±7.0 <sup>b</sup>	104.7±2.4 <sup>b</sup>	0.33±0.04 <sup>b</sup>	0.24±0.02 <sup>b</sup>	5.5±0.4 <sup>b</sup>
Streptozocin + melatonin <sup>3</sup>	6.25±0.5 <sup>a</sup>	57.0±4.2 <sup>a</sup>	45.3±3.1 <sup>a</sup>	0.81±0.11 <sup>a</sup>	1.05±0.11 <sup>a</sup>	10.0±0.8 <sup>a</sup>

<sup>1</sup>For details of the experimental conditions and definition of units see the text; <sup>2</sup>Data are expressed as mean±SEM of 8 rats in each group. In each column, figures bearing different letter superscripts are significantly different at  $P<0.05$  (one way ANOVA and LSD test); <sup>3</sup>This group of rats were treated by gavage with 5 mg melatonin/kg body weight per day for a period of 15 days prior to the administration of streptozocin; ND=not determined

measured on heparinised blood that was obtained from the animals which fasted for 24 hours. Enzymatic kits (Pars Azmoon Company, Tehran, Iran) were prepared according to the procedures of Barham and Tindler,<sup>29</sup> Allain et al.<sup>30</sup>, and Fossati and Prencipe,<sup>31</sup> respectively.

## Results

The effects of melatonin gavage pre-treatment on plasma glucose, cholesterol and TG levels as well as the activities of hepatic hexokinase, glucokinase and G6PD in streptozocin injected rats are shown in Table 1. As seen, diabetic animals demonstrated statistically significant increases in plasma glucose, cholesterol, and TG levels compared to normoglycemic animals. The activities of hexokinase, glucokinase and G6PD decreased significantly in streptozocin diabetic animals compared to the normal control. Gavage pre-treatment of animals with melatonin for 15 days, prior to streptozocin injection, protected the liver against some of the ill effects of streptozocin. Fasting blood glucose, cholesterol, and TG stayed at levels observed for the normal control group. The activities of the three enzymes of carbohydrate metabolism in melatonin pre-treated animals showed no significant difference with the values observed for normoglycemic rats. Pre-treatment of

normoglycemic animals with melatonin did not affect G6PD and hexokinase activities in the liver, but significantly decreased the activity of hepatic glucokinase (Table 1).

Table 2 demonstrates the effects of melatonin post-treatment on the same set of parameters in streptozocin-diabetic rats. As noted, melatonin post-treatment of streptozocin-diabetic rats normalized the levels of fasting blood glucose, serum cholesterol and TG. It also increased the specific activities of hepatic hexokinase, glucokinase and G6PD to the levels observed in normal livers.

The effects of pre- and post-treatment of streptozocin injected with melatonin rats on OGTT are shown in Tables 3 and 4, respectively. As indicated, both pre-and post-treatment with melatonin improved glucose tolerance in streptozocin injected animals.

## Discussion

It was demonstrated by previous investigators<sup>17</sup> that administration of melatonin to pinealectomized rats resulted in increased insulin secretion from pancreatic  $\beta$ -cells and also in decreased plasma glucose. In addition, it was found that the number of insulin receptors on hepatocyte membranes increased significantly

**Table 2.** Effects of melatonin post-treatment on fasting blood glucose, cholesterol, triacylglycerol (TG), hepatic hexokinase, glucokinase and G6PD activities in streptozocin-induced diabetic rats

Experimental groups <sup>1</sup>	Fasting blood glucose (mM)	Plasma cholesterol (mg/dL)	Plasma TG (mg/dL)	Hexokinase (mu/mg protein)	Glucokinase (mu/mg protein)	G6PD (mu/mg protein)
Normoglycemic control	6.3±0.2 <sup>2a</sup>	54.3±3.2 <sup>a</sup>	55.4±3.9 <sup>a</sup>	0.94±0.07 <sup>a</sup>	1.24±0.18 <sup>a</sup>	10.2±0.6 <sup>a</sup>
Diabetic control	20.5±0.6 <sup>b</sup>	121.3±5.4 <sup>b</sup>	110.9±1.7 <sup>b</sup>	0.30±0.01 <sup>b</sup>	0.24±0.03 <sup>b</sup>	5.2±0.4 <sup>b</sup>
Diabetic +melatonin <sup>3</sup>	8.0±0.4 <sup>a</sup>	56.3±3.5 <sup>a</sup>	60.7±1.7 <sup>a</sup>	0.80±0.06 <sup>a</sup>	1.09±0.12 <sup>a</sup>	10.3±0.8 <sup>a</sup>

<sup>1</sup>For details of the experimental conditions and definition of units see the text; <sup>2</sup>Data are expressed as mean±SEM of 8 rats in each group. In each column, figures bearing different letter superscripts are significantly different at  $P<0.05$  (one way ANOVA and LSD test); <sup>3</sup>One week after streptozocin administration and establishment of a fasting blood glucose level above 17.0 mM in diabetic animals, this group of rats were treated by gavage with 5 mg melatonin/kg body weight/day for a period of 15 days

**Table 3.** Effect of melatonin pre-treatment on OGTT in streptozocin injected rats

Experimental groups <sup>1</sup>	Plasma glucose (mM)			
	Zero time <sup>2</sup>	45 min	90 min	135 min
Normoglycemic control	6.7±0.20 <sup>3</sup>	8.8±0.06	5.8±0.43	5.7±0.29
Diabetic control	19.1±1.29	23.5±1.98	21.3±2.40	19.7±1.24
Streptozocin+melatonin	6.2±0.50*	11.8±0.17*	9.1±0.49*	8.0±0.49*

<sup>1</sup>For details of the experimental conditions, please refer to the text and to reference 28; <sup>2</sup>Zero time glucose concentration refers to plasma glucose concentration of animals at the end of the experimental period after 24 hours of fasting prior to glucose feeding<sup>28</sup>; <sup>3</sup>Data are expressed as mean±SEM of 8 rats in each group. Figures bearing asterisks (\*) are significantly different from their corresponding values in the diabetic control group at  $P<0.05$  (one way ANOVA and LSD test)

upon melatonin treatment. Other investigators demonstrated decreased insulin receptors and increased glucagon receptors on the hepatocyte membranes of diabetic pinealectomized rats.<sup>18</sup> We have demonstrated that the specific activity of hepatic glucokinase, an insulin-inducible enzyme<sup>32</sup> and the major enzyme for the phosphorylation of glucose in the liver decreases about six fold in diabetic animals and both pre- and post-treatment of streptozocin injected animals returns glucokinase activity to the values observed in normal controls (Tables 1 and 2). This is in agreement with the effect of another antioxidant, quercetin,<sup>24</sup> and also the effect of the aqueous extract of *Teucrium polium*<sup>26</sup> (a plant extract with several antioxidants) on the activity of this enzyme in the liver. It appears that melatonin acts in the capacity of an antioxidant both in scavenging free radicals produced by streptozocin and also via its effect on increasing insulin secretion by  $\beta$ -cells.<sup>17</sup> It also has an augmenting effect on the number of insulin receptors on hepatocyte membranes.<sup>18</sup> Such effects result in the normal induction of liver glucokinase. Decreased glucokinase activity of normoglycemic animals pretreated with melatonin may be due to disturbances in the insulin signaling of such animals by melatonin as noticed for another antioxidant<sup>23</sup> (Table 1). We did not observe any significant difference between the specific activities of hepatic glucokinase in the melatonin pre- and post-treatment groups.

Our results of both melatonin pre- and post-treatment on the specific activity of liver G6PD (Tables 1 and 2) agrees with the work of Anwar and Meki<sup>14</sup> on the effects of this antioxidant on the

enzyme in the liver and brain of diabetic rats.

The plasma glucose lowering effect of melatonin and also its effect in diminishing plasma TG and cholesterol levels in diabetic animals (Tables 1 and 2) agree with previously reported investigations.<sup>19,20</sup> National Diabetes Data Group (NDDG) has devised certain criteria for diagnosing diabetes mellitus.<sup>33</sup> One of these criteria states that a plasma glucose greater than 11.1 mM at two time points (2 hours and an earlier time point) during a glucose tolerance test is indicative of diabetic glucose tolerance. Our streptozocin-induced diabetic animals (Tables 3 and 4) satisfy this criterion. NDDG proposes that if plasma glucose exceeds 11.1 mM at least once between 0 and 2 hours during a glucose tolerance test, and eventually falls to 7.8 – 11.0 mM by 2 hours, it is indicative of impaired glucose tolerance. Both our melatonin pre- and post-treatment groups fall in this category (Tables 3 and 4). Therefore, it could be concluded that although melatonin pre- or post-treatment of streptozocin administered animals improves glucose tolerance curves, it does not completely normalize them.

In conclusion, melatonin pre- and post-treatment of streptozocin administered rats not only lowers plasma glucose, cholesterol and TG, but also affects carbohydrate metabolism in hepatocytes through an increase in the specific activities of glucokinase, hexokinase and G6PD, the key enzymes of carbohydrate metabolism in such cells.

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**Table 4.** Effect of melatonin post-treatment on OGTT of streptozocin-induced diabetic rats

Experimental groups <sup>1</sup>	Plasma glucose (mM)			
	Zero time <sup>2</sup>	45 min	90 min	135 min
Normoglycemic control	6.3±0.25 <sup>3</sup>	8.4±0.18	6.8±0.14	6.7±0.35
Diabetic control	20.5± 0.56	23.4±0.8	21.6±1.13	20.5±1.26
Diabetic + melatonin	8.0±0.36*	11.5±0.2*	9.6±0.15*	8.1±0.05*

<sup>1</sup>For details of the experimental conditions refer to the text and to reference 28(See comment in Table 3.); <sup>2</sup>Zero time glucose concentrations refer to plasma glucose concentrations of animals at the end of the experimental period after 24 hours of fasting prior to glucose feeding<sup>28</sup>; <sup>3</sup>Data are expressed as mean±SEM of 8 rats in each group. Figures bearing asterisks (\*) are significantly different from their corresponding values in the diabetic control group at  $P<0.05$  (one way ANOVA and LSD test)

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