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Original Research Article

An Experimental Study to Evaluate the Effect of Melatonin on Reactive Oxygen Species (ROS) in Streptozotocin Induced Diabetes in Rat Model

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Conflict of interest: Nil

Abstract:

Background: Diabetes mellitus, a metabolic dysfunctional group comprises of chronic hyperglycemia arising either due to defective insulin secretion, insulin action or both. The prevalence of diabetes is directly proportional to increasing age. The present study involves assessing the impact of Melatonin, and its combination with Biguanide (Metformin) on Reactive Oxygen Species in blood of rats with diabetes induced by Streptozotocin.

Aim: To evaluate the effect of Melatonin and its combination with Metformin on Reactive Oxygen Species (ROS) in Streptozotocin induced T2DM rat model.

Methodology: The current experimental investigation was carried out at the Department of Pharmacology and Therapeutics, King George's Medical University, Lucknow. Adult male Wistar Rats were randomly divided into 5 groups, each group containing (n=6 rats) and assessed for 65 days. The study parameter used for assessment was- Reactive Oxygen Species (ROS) in response to monotherapy with Melatonin and Metformin and in combination with both Melatonin and Metformin in Streptozotocin induced diabetic rats.

Results: The mean ROS level was assessed at Day 0 (Baseline), Day 36 and Day 65 (Final reading). On Day 65, the reduction in mean ROS was observed in all treatment groups, but statistically significant reduction was observed in combination therapy group (Metformin and Melatonin) as compared to baseline on Day 0 and to Group B (Diabetic Control) on Day 36.

Conclusion: Based on current results the study concludes that Melatonin and combination with Metformin proved to be beneficial in restoring study parameter in blood samples of Type 2 Diabetes Mellitus in rats.

Keywords: Melatonin, Metformin, Reactive Oxygen Species (ROS), Streptozotocin, Wistar Rat.

Introduction

Diabetes mellitus is a collection of metabolic disorders characterized by persistently high blood sugar levels that result from either ineffective insulin secretion, ineffective insulin action, or both. As people age, the prevalence of diabetes mellitus increases in a direct proportion. Three Ps: polyphagia, polydipsia, and polyuria, make up the diabetes triad. Depending on the type of diabetes, uncontrolled diabetes can result in consequences such stupor, coma, ketoacidosis, or hyperosmolar non-ketotic syndrome. [1]

Patients living with diabetes who experience multiorgan complications, namely microvascular and microvascular problems, have higher rates of early morbidity and mortality.[2] The World Health Organization (WHO) reports that 1.6 million deaths globally are attributable to type 2 diabetes. Furthermore, 98% of diabetes cases internationally are diagnosed with type 2 diabetes. Even though type 2 diabetes usually develops later in life, there are still significant worries about the growth in diabetes prevalence due to the rising rate of juvenile obesity. [2] According to the International Diabetes Federation, 419 million adults in the 20–79 age range had diabetes in 2015. By 2040, the prevalence is predicted to rise from 8.8 to 10.4%, impacting over 640 million people globally. [3]

India only behind China in the number of diabetes, with 77 million. India is predicted to have 134.2 million cases of diabetes mellitus by the end of 2045. (2) Diabetes mellitus is a disease of lifestyle. The pathogenesis of type 2 diabetes mellitus (T2DM) is largely caused by lifestyle variables such as alcohol use, cigarette smoking, sedentary lifestyles, and physical inactivity. T2DM development has been mostly attributed to obesity.[4]. Even if changing one's lifestyle can help avoid type 2 diabetes, Biguanides like metformin

are still the gold standard for therapeutic drugs used to treat the disease. The Diabetes Prevention Program: a randomized clinical trial showed that, in comparison to the placebo group, metformin medication plus an intensive lifestyle intervention significantly slowed the progression of type 2 diabetes by 31% and 58%, respectively. [5].

Free radicals are created when the blood is exposed to elevated glucose levels for an extended period of time. They are exceedingly unstable and highly reactive species because they are chemical entities that contain one or more free unpaired electrons. They cause oxidative stress [6] and modify gene expression, both of which are significant contributors to microvascular and cardiovascular diabetes problems. Oxidative free radical production triggers pro-inflammatory pathways, which in turn raise the levels of inflammatory mediators. Additionally, mitochondrial ROS proliferate as a result of insulin resistance. [7].

The pineal glands release melatonin, albeit they do not do so only [8]. Pinealocytes synthesis this hormone from the amino acid tryptophan. Therefore, N-acetyl, 5-methoxytryptamine, an indole derivative of tryptophan with a flexible peptide-like chain linked to the C3 position, is the molecular basis of melatonin [9]. Melatonin production and secretion are triggered by darkness and suppressed by light. It is also referred to as the "hormone of darkness" [10]. It starts to secrete at or after sunset and peaks between 12 and 2 am in the middle of the night. The majority of the melatonin in the blood, which ranges from 80 to 120 pg/ml, is produced at night. During the day, its serum concentrations drop to 10–20 pg/ml. [11]. Furthermore, melatonin's activity on its receptors is responsible for the circadian production of insulin by the pancreatic islets, both in vivo and in vitro. In the past few years, research employing pinealectomized rats and animal models with melatonin receptor knockouts has been carried out to illustrate the function of melatonin in glucose metabolism. [12].

Research has indicated that the pathophysiology of type 2 diabetes mellitus involves signals from melatonin receptors [13]. A possible correlation has been observed between the release of melatonin into the bloodstream, glucose secretion and absorption, and insulin secretion by the pancreatic beta cells [14]. Melatonin levels have decreased with increased insulin resistance, indicating a known functional antagonistic relationship between melatonin and insulin release. [15]. For substances like hydroxyl and peroxide free radicals, melatonin also functions as a strong antioxidant and free radical scavenger [16]. Melatonin increases the activity of endogenous antioxidants such as glutathione peroxidase, catalase, and superoxide dismutase through its receptor-mediated effect. Exogenous melatonin injection before inflammatory disorders such as diabetes has been shown in animal experiments to reduce pro-inflammatory cytokines and inflammatory responses [10].

This study was designed to assess the anti-oxidative effect of melatonin and metformin in blood samples in respect to reactive oxygen species (ROS) using dye tracking method by vital dye.

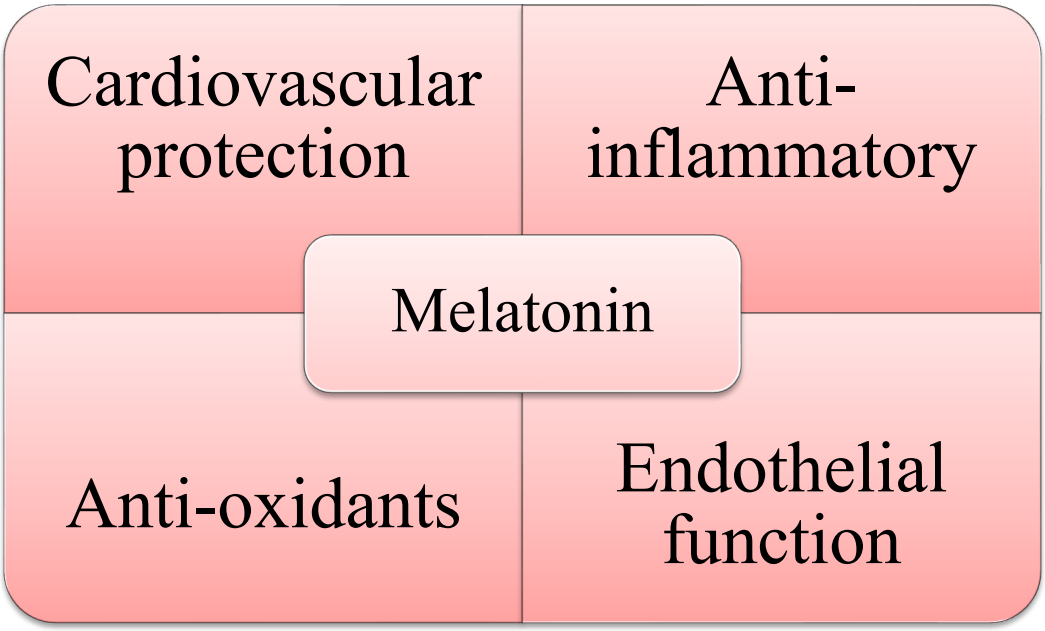

Figure 1: Physiological action of Melatonin on various tissues and functions

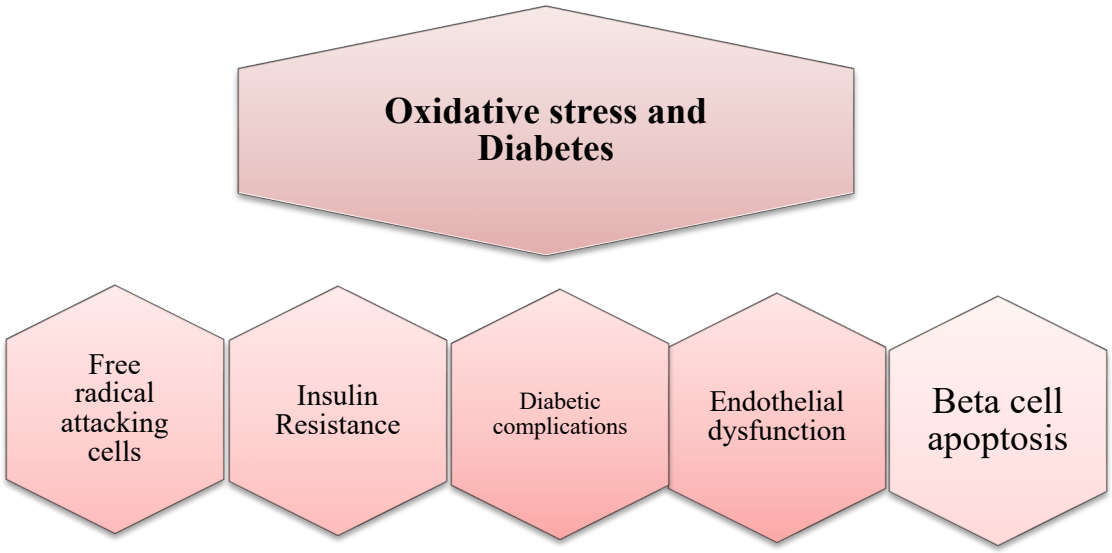

Figure 2: Effect of oxidative stress in Diabetes mellitus type 2

Material and Methods

This study was conducted in the department of Pharmacology and Therapeutics, King George's Medical University, Lucknow to evaluate the Effect Of Melatonin on Reactive Oxygen Species in Streptozotocin Induced Diabetes in Rat Model after obtaining clearance from the Institutional Animal Ethics Committee (IAEC) vide ethical clearance **Project no. 178/IAEC/2022.**

Experimental Animals

The experiments were conducted on adult Male Wistar rats weighing 150-200 grams. The animals were housed in Institutional Animal House under standard conditions of housing that is room temperature of 24-27° C and humidity of 60-65 % with 12-hour light and dark cycle. The food in the form of dry pellets was given and water was available ad libitum. For present study, 30 experimental animals (Male Wistar Rats) were procured from CSIR-IITR, Gehru Campus, and Lucknow, India.

A total number of 30 adult male Wistar rats weighing 150-200 grams were included in the study. The animals were housed in Institutional Animal House under standard conditions of housing that is room temperature of 24-27° C and humidity of 60-65 % with 12-hour light and dark cycle. The food in the form of dry pellets was given and water was available ad libitum.

The rats were allowed to acclimatize to the new environment for a period of one week in cages. After acclimatization, rats were divided randomly into 5 groups from A to E. Each group contained n=6 rats. Rats in Group A were maintained on normal pellet diet (NPD) and water ad libitum while rats in remaining groups (B to E) were given high fat diet (HFD). Normal pellet diet (NPD) and High-fat diet (HFD) were procured from Bharat Science Solution Company, Lok Nagar, Unnao, and Uttar Pradesh. The high-fat diet (HFD) comprised of 58% fat, 27.5% carbohydrate and 14.5% protein, as a percentage of total kcal.

Drugs and Chemicals: Melatonin was obtained from Dr. Morepen (India). Metformin was purchased from Cipla Ltd (India) and Streptozotocin was procured from SRL Laboratories (India). All additional chemical substances were of superior analytical quality.

Induction with Type 2 Diabetes Mellitus

Animals were fed with high fat diet (HFD) for a period of 28 days except Group A (Normal control). After 28 days of high fat diet all rats were fasted from 7 am to 3 pm. Rats on normal pellet diet were given citrate buffer and rats on high fat diet were given a single intraperitoneal injection of Streptozotocin (STZ) in dose of 60 mg/kg body weight. STZ at a dose of 60 mg/kg was prepared in cold citrate buffer (pH 4.5, 0.1 M) [20] and given intraperitoneally. Rats were stabilized after one week of STZ injection. After one week, blood samples were taken by tail vein and their random blood glucose levels were checked using glucometer (Dr. Morepen). Rats with random blood glucose level >200 mg/dL were considered diabetic rat and used in the study. [21]

Experimental Design

A total of 30 adult male Wistar rats were enrolled in our study. Rats were randomly divided into 5

groups from A to E. Each group had 6 rats each and were assessed for 28 days. Group A served as Normal control group where the rats were fed with NPD and water ad libitum, throughout the study. Also, they were administered citrate buffer solution orally, to serve as control group. The rats in Group B were fed with HFD and water ad libitum, throughout the study hence served as diabetic control group. Metformin treated group C rats were given Metformin at dose of 100 mg/kg body weight, per oral, dissolved in normal saline by oral gavage with HFD and water for 28 days. [22] [23] Rats treated with test drug Melatonin in group D were given Melatonin at dose of 15 mg/kg body weight, per oral, dissolved in normal saline by oral gavage with HFD and water for 28 days. [24] [25] Group E was combination therapy group where rats were administered both Metformin at dose of 100 mg/kg body weight and Melatonin at dose of 15 mg/kg body weight, per oral, dissolved in normal saline by oral gavage with HFD and water for 28 days.

All the drugs were administered orally after dissolving them in normal saline for a period of 28 days after a period of seven days after administration of STZ injection. Group A and Group B were administered only 1 mL of Normal saline per oral. Readings were taken on Day 0 (Baseline), Day 36, and Day 65 (Final reading) for ROS assessment in blood samples.

Flow Chart of Experimental Protocol

Total animal groups = 5

Each group contains 6 Wistar rats (n = 30)

Flow Chart 1:

- RBS: Random Blood Glucose
- ROS: Reactive Oxygen Species
- STZ: Streptozotocin
- NPD: Normal Pellet Diet
- HFD: High Fat Diet

Procedure

Estimation of Blood glucose

Glucose levels were estimated to find if the rats were diabetic or not. It was measured by a glucometer (Dr. Morepen) with a drop of blood from tail vein.

Collection of Blood samples by Intra-cardiac puncture (on Day 65)

Thiopentone sodium, an anesthetic, had to be administered intraperitoneally via a 5 mL syringe fitted with a 24G needle at a dose of 40 mg/kg for this procedure. This procedure was done on the last day (Day 65) of the study to obtain blood samples. The rat was given anaesthesia, and the extent of the sedation was demonstrated by the rat's slow breathing, lack of spontaneous movement, and lack of reaction to stimuli (pinching a toe).

The anesthetized animal was turned to face away and laid on its back. The lowest rib was used to hold the syringe at a 45-degree angle once the estimated position of the heart was felt. The syringe barrel was filled with blood after the needle was placed between two ribs. It took about 3–4 mL of blood to draw. Thiopentone sodium overload caused the animal to be put to death right away. [26]

Estimation of Reactive Oxygen Species

- The blood samples were centrifuged at 3000 rpm for 2 minutes at 4°C in flow cytometer.
- The cells were stained by a vital dye called the **CellROX deep red** (cellular oxidative stress detection) manufactured by Life Technologies Corporation.
- The catalogue number was C 10422 and LOT number 2059229 from Invitrogen Thermo Fisher Scientific.
- The staining was done using 2.5 µm dye for 30 minutes.
- The cells were washed with PBS (Phosphate Buffer Saline) and analysed immediately.

Statistical analysis

The collection of data was organized and tabulated in Microsoft Excel (Microsoft Office 365) and statistical analysis was done using SPSS (Statistical Package for Social Sciences) version 23.0 Statistical Analysis Software.

The values were represented in Number and Mean ± SD. The change in groups mean at three-time intervals were calculated using the paired t-test. Intergroup variations were assessed employing one-way ANOVA, followed by subsequent post-hoc analysis (Tukey-HSD test) was employed to assess individual group arrangements among others. The p-value of less than 0.05 was considered statistically significant.

Results and Observations

In this study, a total of 30 rats were randomly allocated to five study groups and were evaluated for the effect of monotherapy with Melatonin and Metformin and their combination therapy to assess reactive oxygen species (ROS), in blood samples of streptozotocin induced diabetic rats.

Intergroup Comparison of Reactive Oxygen Species (ROS) at different time intervals:

On Day 0 (Baseline) non-significant change in mean values of Reactive Oxygen Species (ROS) was observed in blood among all Groups from A to E. On Day36, diabetic control Group B and all other therapeutic Groups C to E showed statistically significant change in mean blood ROS levels as compared to normal control Group A. However, all therapeutic Groups C to E showed statistically significant reduction in blood ROS levels as compared to diabetic control Group B on Day 65.

On Day 65, a statistical decline in blood ROS was observed maximally for combination therapy Group E (-19.12±3.8) followed by monotherapy with Melatonin Group D (-17.30±0.27) and monotherapy with Metformin Group C (-7.69±3.21) as compared to diabetic control Group B from Day 36.

Discussion

Comparison of ROS values among therapeutic groups (Monotherapy and Combination Therapy) and controls

On Day 0 (Baseline) non-significant change in mean values of Reactive Oxygen Species (ROS) was observed in blood among all Groups from A to E. On Day36, diabetic control Group B and all other therapeutic Groups C to E showed statistically significant change in mean blood ROS levels as compared to normal control Group A.

However, the rats in monotherapy with Melatonin, Group D and combination therapy Group E did not show statistically significant difference in mean ROS levels in blood compared to normal control Group A on Day 65. No significant difference in mean blood ROS levels were noted between rats of therapeutic Groups C to E and diabetic control rats Group B on Day 36.

However, all therapeutic Groups C to E showed statistically significant reduction in blood ROS

levels as compared to diabetic control Group B on Day 65. Thus, both monotherapy with Melatonin and combination therapy with Metformin and Melatonin showed evident action in lowering mean blood ROS levels as compared to diabetic control rats. Tukey HSD test to report group differences showed no statistically significant difference among therapeutic Groups C to E on Day36.

However, on Day65, a statistically significant difference was observed between Groups C to E as compared to Group B, but no significant difference was observed between Groups D (Monotherapy with Melatonin) and E (Combination therapy). This means that treatment with Melatonin as monotherapy brought the mean blood ROS levels near to that of treatment with Combination therapy.

On Day65, mean blood ROS in Combination Therapy Group E was (10.79±1.90) which was closest to normal control Group A (9.58±1.31). While, Group D (Melatonin monotherapy) had a mean blood ROS value of (13.30±1.23) and Group C (Metformin monotherapy) showed a mean blood ROS value of (22.14±5.39).

Thus, treatment with Combination therapy were able to show a better reduction in blood ROS levels as compared to monotherapy with Melatonin and Metformin. Between treatment with Metformin and Melatonin, rats in monotherapy with Melatonin exhibited a better decline in blood ROS as compared to rats in monotherapy with Metformin as used in our study. Alyaa Farid et. al., (2022) have mentioned in their work that Melatonin has demonstrated to decrease the expression of NF-κB in streptozotocin induced diabetes in rats. They also observed a significant reduction in ROS parameters and inflammatory mediators. Oxidative damage was less in melatonin treated group as compared to the diabetic control group. Similar findings are observed in our study too. [26] Similar results have been obtained by Banerjee, A., et al, (2021) where combination therapy with Metformin and Melatonin was used which helped in alleviation of excess ROS produced due to oxidative stress in high fat fed Sprague Dawley rats. [25]

According to a study done by Espino J. et al, (2011), Melatonin exerts a neuroprotective action by quenching free radicals formed during a chronic inflammatory state as in T2DM and hence decrease the production of ROS. They also stated that Melatonin effectively scavenged production of hydroxyl radicals and inhibit hydroxyl radical lipid peroxidation in liposomes. The same result was observed in our study too. [15]

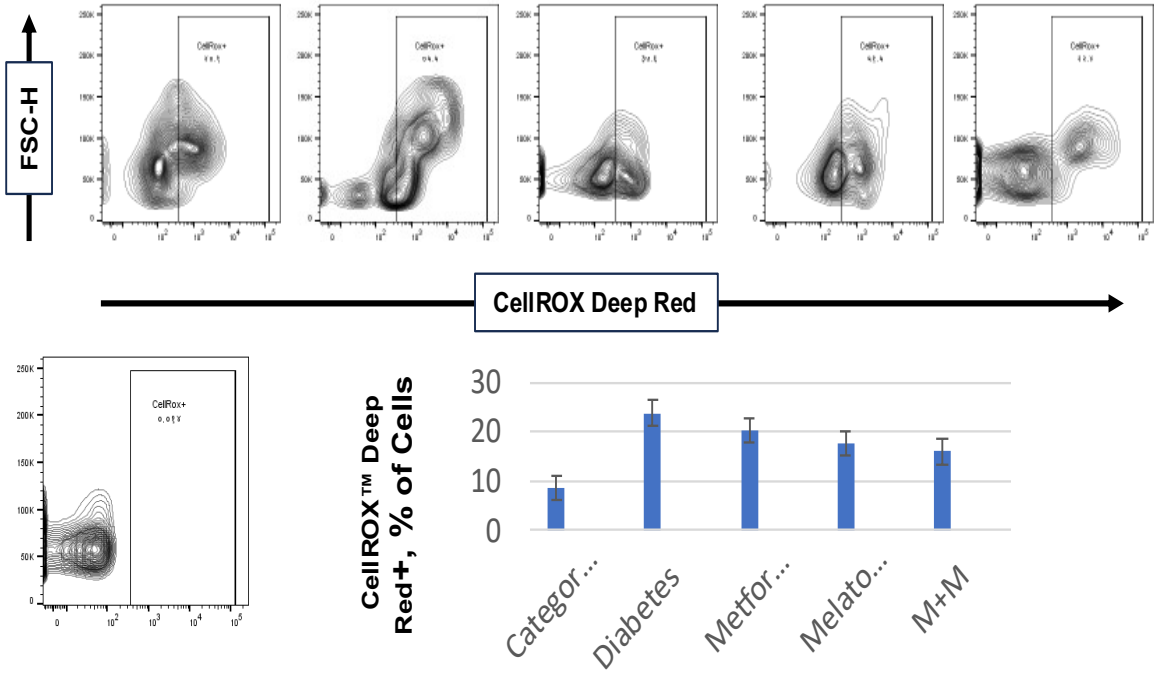

Figure 3: Action of CellRox deep red vital dye on reactive oxygen species on rat's blood sample.

Conclusion

The present study aimed to evaluate the effect of Melatonin on Reactive Oxygen Species (ROS) in Streptozotocin induced diabetes in rat model. Beneficial effects of Melatonin as monotherapy have been observed as it directly scavenges free radicals by alleviating oxidative damage and reduces generation of free radicals as compared to the standard drug.

Metformin showed only some effect. Significant results were obtained when Melatonin and Metformin were administered in combination, limiting the oxidative damage associated with T2DM in blood level.

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