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Research Article
Protective Role of Melatonin in Streptozotocin Induced Pancreatic Damages in Diabetic Wistar Rat

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Abstract

Background and Objective: Hyperglycemia is a representative hallmark and risk factor for diabetes and is closely linked to diabetes associated complications. The aim of the present study was to evaluate the therapeutic potential of exogenous melatonin against the streptozotocin induced pancreatic damages in rats. **Materials and Methods:** Streptozotocin was injected for consecutive 6 days. Diabetes was confirmed by blood glucose measurement after 72 h and on 7th day after injection. Animals having blood glucose level above 250 mg dL^{-1} were considered as diabetic and were administered exogenous melatonin for 4 weeks. Animals were euthanized after last dose, pancreas were dissected out, weighed and fixed in Bouin's fixative for histology and further tissues were kept at -20°C for biochemistry. **Results:** Diabetic rats displayed significant increase in lipid peroxidation, but pancreatic weight index, antioxidant system (GSH, SOD and CAT) showed decrease. Melatonin treatment to diabetic rats restored the alteration in physiological and biochemical markers. Results were supported by the histopathological observations, STZ treated pancreas showed damage in islets of langerhans, while as melatonin treated diabetic rats recovered the cellular architecture which inturn normalize the function of the pancreas. **Conclusion:** Therefore, melatonin might be considered as a molecule to protect the pancreatic damages.

Key words: Streptozotocin, hyperglycaemia, melatonin, diabetes, pancreatic damage, diabetic rats

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Diabetes is a group of metabolic disorder in which there occurs high blood sugar level over a prolonged period[1]. It is one of the familiar causative key factor of mortality in the developing countries where it affects more than 170 million individuals in the whole world[2]. The key diagnostic feature of diabetes is hyperglycaemia and disturbed metabolism of carbohydrates, lipid and proteins caused by either insulin deficiency or improper insulin action or both. Signs and symptoms of diabetes includes polyurea, polydipsia and polyphagia. Serious long term complications include heart disease, stroke, chronic kidney failure, liver diseases, foot ulcers and damage to the eyes[3].

Streptozotocin is a common diabetogenic molecule to induce diabetes, due to its evident properties such as beta cell cytotoxic, oncolytic, oncogenic and antibiotic properties[4]. Hyperglycaemia and oxidative stress are two well-known cause of the etiology and pathogenesis of disease complications. Its administration develops a good diabetic model for associated research. Glycemic control is important in diabetes mellitus to minimize the progression of the disease and the risk of potentially devastating complications. To the best of author's knowledge this is the first experimental study to show induction and generation of diabetic rats model at very low dose (15 mg kg^{-1} b.wt. for 6 days). The significance of low dose is that it prevents the complete degeneration of beta cells[5,6]. Till date 30, 40, 35, 45, 50, 60 and upto 180 mg kg^{-1} b.wt. were used to induce diabetes[7,8]. Streptozotocin causes depletion of the intracellular nicotinamide dinucleotide (NAD) islet cells. Streptozotocin also induces DNA strand breaks and methylation in the pancreatic islet cells. The STZ is also related with the generation of reactive oxygen species causing oxidative damage to the pancreatic islet cells[9]. The known underlying mechanism for the beta cell death is the auto-antibody generation, these auto-antibodies in turn acts on the beta cells hence resulted in cell death[10]. Damage in pancreas is associated with the development of the diabetes. Pancreatic islet cells destruction may lead to drop in insulin secretion and increase in blood glucose concentration[11]. Excess glucose becomes auto-oxidised and becomes the source of reactive oxygen species. These ROS causes cellular damages and cell loses its normal physiological homeostatic mechanism[12]. Previous it has been reported that during diabetes the food and water intake increases, but body weight decreases[13]. Now-a-day, anti-oxidants are now freely available which includes vitamin-E, C, plant extracts and some synthetic anti-oxidant molecules like melatonin[14]. The implication of anti-oxidant might be act protective agent against reactive oxygen species mediated islet cell damage[15].

Melatonin is an endogenous neurohormone secreted by the pineal gland in mammals, it is an indoleamine (N-acetyl-5-methoxytryptamine)[16,17]. Synthesis of melatonin is amplified in darkness and suppressed when animals are exposed to light. It is mainly synthesized and secreted by the pineal gland, but 25% of melatonin production is of extra-pineal sites[18]. It participates in different physiological processes such as regulation of reproduction, circadian rhythms[19,20], antioxidative role[21,22], oncostatic[23], anti-inflammatory property[24], immunomodulator and regulator[25,26] and neuroprotective role[27]. Now-a-days melatonin is known as the most powerful scavenger of various ROS, like as hydroxyl and peroxyl radicals[28,20]. Melatonin has very peculiar property in comparison to the other antioxidants that it crosses all morphological barriers, i.e., the blood-brain and the placenta and is well distributed throughout all cells[28,20]. Therefore, present study was hypothesized to elucidate the importance and impact of melatonin during diabetic state in order to evaluate the therapeutic potential of exogenous melatonin (MEL) on pancreas.

MATERIALS AND METHODS

This experimental study was executed during the month of January-April, 2018 at Department of Zoology, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India.

Chemicals, reagents and instruments: Streptozotocin (STZ), Melatonin (Mel), citrate monohydrate, Sodium citrate Thiobarbituric acid (TBA), Tris-hydrochloric acid (Tris-HCl), Phosphoric acid and Butylated Hydroxy Toluene (BHT), Glutathione reduced (GSH), Phenazine methosulphate (PMS), Glacial acetic acid, H_2O_2, Dithio-bis 2-nitrobenzoic acid (DTNB), Nitroblue tetrazolium salt (NBT), Nicotine Adenine Dinucleotide Phosphate (NADPH) of analytical grade procured from Sigma Aldrich, USA and Himedia limited, India. ELISA C- peptide kit procured from Crystal Chem Inc.; cat. No. 90060; Downers Grove). Centrifuge (Remi C-24BL) and Perkin Elmer UV-Visible Spectrophotometer (LAMBA, Serial No. 501812090010) and ELISA reader, TECAN. All the chemical and reagents utilized are of analytical grade.

Animal maintenance: Male albino rats of Wistar strain weighing approximately 180±10 g of same age groups were procured from Defence Research and Development Establishment (DRDE) Gwalior, M.P. India. The Animals were maintained under standard temperature and light controlled room, humidity and housed six per cage. The rats were acclimatized for a week before the commencement of the experiment. All animal experimental procedures were

approved by the Animal Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) under the Institutional Animal Ethics Committee at SLT institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur, Chhattisgarh, India. All experimental procedures were performed in accordance with the national and international guidelines and regulations approved by SLT institute of Pharmaceutical Sciences, Guru Ghasidas Vishwavidyalaya, Bilaspur Institutional Animal Ethics Committee (Reference No. 157/IAEC/Pharmacy/2016).

Induction and confirmation of diabetes: Streptozotocin (STZ) was prepared in 0.1 m citrate buffer (pH 7.4) (15 mg kg^{-1}) and was injected intraperitoneally for six consecutive days. Blood glucose level of the animals were monitored using Glucometer (ACCUCHECK) after 72 h of streptozotocin treatment. Rats with blood glucose level exceeding higher than 250 mg dL^{-1} upto 6th day were confirmed as diabetic. Animals were divided into different groups and were kept for experimentation for 4 weeks as under the following experimental design.

Experimental design:

Group I : Normal control rats
Group II : Diabetic control (STZ 15 mg kg^{-1} b.wt., 6 days, i.p.)
Group III : STZ+MEL [STZ 15 mg kg^{-1} (6 days)+1 mg kg^{-1} b.wt., 4 weeks]
Group IV : MEL (1 mg kg^{-1} b.wt., 4 weeks)
Group V : STZ+GB (STZ 15+0.5 mg kg^{-1} b.wt., 4 weeks, p.o.)
Group VI : GB (0.5 mg kg^{-1} b.wt., 4 weeks)

The animals were sacrificed after last dosage using ether anaesthesia. Blood was collected directly from the heart by cardiac puncture. Pancreatic tissues were dissected out, weighed, fixed in Bouin's fixative for histopathological studies and further tissues were stored at -20°C for tissue biochemistry.

Assessment of weight: The body weight of the animals were checked before commencement of the experiment and also weekly during the experiment until sacrifice. The weight of pancreas was assessed after the sacrifice. Pancreatic liver index was calculated for each animal using the following equation:

$$\text{Pancreatic index (\%)} = \frac{\text{Pancreas weight}}{\text{Body weight}} \times 100$$

Tissue biochemistry: Lipid peroxidation assay was done according to the method[29]. The homogenate was prepared by Tris-HCl buffer and absorbance was measured at 535 nm. Reduced glutathione content was measured following modified method[30]. The absorbance of the yellow coloured supernatant was measured at 412 nm. Molar extinction coefficient of 13,100 was used to calculate GSH content. Superoxide dismutase (SOD) activity was recorded by modified method[31] and absorbance was taken at 560 nm against the blank. The catalase (CAT) activity was examined by spectrophotometric method[32] with some modifications absorbance was measured at 240 nm for 3 min. Total protein content was quantified by spectrophotometric method[33] with some modifications and absorbance was taken at 625 nm.

C-peptide assay: Blood samples were collected directly from heart in clot accelerator vials tubes and centrifuged at 3000 g for 15 min to collect the plasma. The blood plasma of each group of male rats were stored at -20°C for subsequent C-peptide assay (Crystal Chem Inc.; cat. No. 90060; Downers Grove) the C-peptide analysis was performed according to the instructions provided in the manual of the commercial kit.

Histopathology: Pancreas of the respective groups were harvested and washed with 0.1 M ice cold phosphate buffered saline (PBS) and portion of the pancreas were fixed in Bouin's fixative and paraffin sections of 4-5 mm thickness were cut. Hematoxylin-eosin stained slides were observed under light microscope[34,35] for histopathological changes.

Statistical analysis: Results are expressed as Mean±SE one-way ANOVA was carried out followed by student's test. The $p<0.05$ and 0.01 implied significance[36].

RESULTS

Pancreatic weight index: The pancreatic weight index of diabetic control rats were found decreased when compared to that of the control rats and was found statistically significant ($p<0.05$, 0.01) (Fig. 1). Treatment of exogenous melatonin showed recovery in pancreatic index towards the normal control which was comparable with the standard anti-diabetic hypoglycaemic drug glibenclamide treated rats. The pancreatic indexes of only melatonin treated rats were observed to have normal pancreatic index similar to that of the normal control rats.

Assessment of weekly variations in blood glucose level in individual experimental groups: The weekly blood glucose level was found significantly higher in STZ induced diabetic rats, while as melatonin treated diabetic rats revealed significant restored weekly alterations blood glucose towards the control range (Fig. 2). Melatonin and hypoglycaemic drug does not showed any abnormal change in blood glucose level.

Alterations in blood glucose level between the experimental groups: The assessment of variation in blood glucose level between the groups showed significant increase diabetic rats. However, melatonin administered diabetic rats significantly decrease the blood glucose level and maintained near to control level (Fig. 3).

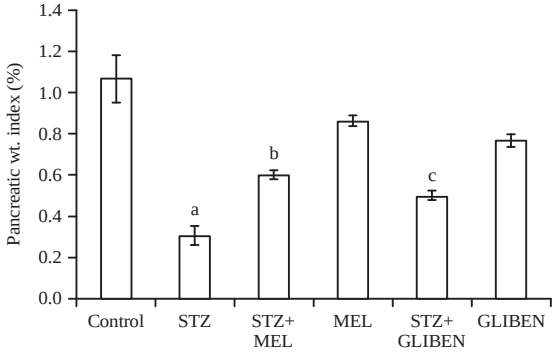

Fig. 1: Pancreatic weight index in different experimental groups. STZ: Streptozotocin, MEL: Melatonin, GB: Glibenclamide

Data are Mean±SE, N= 6, F-value = 29.36, Significant at 5% for ANOVA. [a]STZ vs. CONT at $p \leq 0.05$, [b]STZ vs. STZ+MEL at $p \leq 0.05$, [c]STZ vs. STZ+GB at $p \leq 0.05$

Oxidative stress and antioxidative system (LPO, GSH, SOD and CAT): The STZ intoxicated rats exhibited significant augmentation in LPO and a simultaneous decrease in GSH level in pancreas (F). Increased LPO was expressed in terms of thiobarbituric acid reactive species (TBARS). Exogenous treatment of melatonin (1 mg kg^{-1} b.wt.) significantly decreases LPO and maintained GSH level near to normal pancreas when compared with STZ treated group of rats ($p<0.05$, 0.01). Activities of enzymes were significantly suppressed in STZ intoxicated diabetic rats as compared to normal control ($p<0.05$, 0.01). Exogenous melatonin treatment exhibited significant recovery in the SOD and CAT when compared to STZ induced diabetic control rats ($p<0.05$, 0.01). Maximum recovery was observed in exogenous melatonin treated rats when compared with anti-diabetic drug (Glibenclamide). Alone treatment of melatonin does not reveal any significant alteration in LPO, GSH, SOD and CAT when compared with control (Table 1).

Estimation of total protein content: The STZ induced diabetic rats showed significant decrease in total protein content when compared to non-diabetic control rats. While as diabetic rats treated with melatonin significantly restored towards the total protein content near to control range. Oxidative stress causes the reactive oxygen species (ROS) generation. These ROS causes peroxidation of membrane

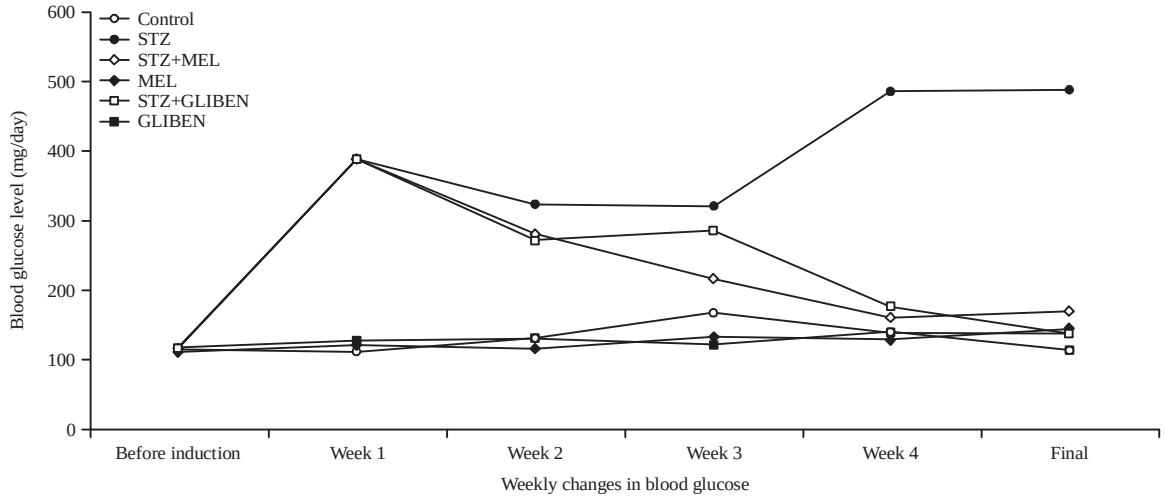

Fig. 2: Weekly changes in blood glucose level in different experimental groups. STZ: Streptozotocin, MEL: Melatonin, GB: Glibenclamide

Data are Mean±SE; N = 6. F-value = 1.51

lipids, integral and transmembrane proteins, hence changes permeability of membrane. Melatonin might have prevented the membrane damage by scavenging the free radicals, hence reverted membrane damage and maintained membrane permeability (Table 1).

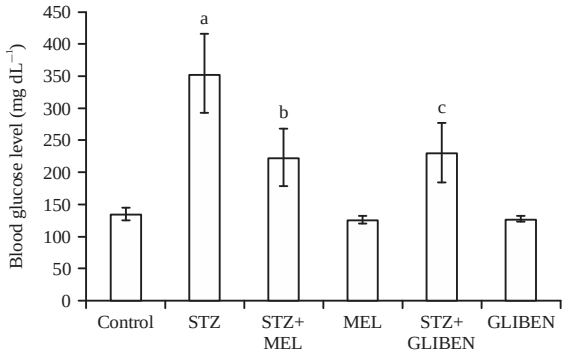

Fig. 3: Mean difference in blood glucose level in different experimental groups. STZ: Streptozotocin, MEL: Melatonin, GB: Glibenclamide

Data are Mean±SE., N = 6. F-value = 7.220, Significant at 5% for ANOVA. [a]STZ vs. CONT at $p \leq 0.05$. [b]STZ vs. STZ+MEL at $p \leq 0.05$, [c]STZ vs. STZ+GB at $p \leq 0.05$

Assessment of C-peptide level: Diabetic control rats revealed significant decrease in C-peptide level, while as exogenous melatonin administration to induced diabetic rats significantly increased the C-peptide level comparable control group of rats. The protective effect of melatonin may be due to the stimulatory and regenerative effect of melatonin on beta cells of pancreas (Table 2).

Histopathology

Pancreas: Pancreas of control group showed histoarchitecture with normal appearance of islets of langerhans well organized endocrine cells, normal cellular environment (Fig. 4a, b). The STZ intoxication causes degeneration, vacuole formation and disintegration of islets of langerhans architecture. Pancreatic injuries showed breakdown of micro-anatomical features such as extensive Beta-cell degranulation, reduced cellular density, border between endocrine and exocrine part becomes invisible, beta cells are degenerated and vacuole formation was observed in STZ induced cellular damage. In diabetic control group of rats diffused and necrotic changes and shrinkage in the islets of langerhans (Fig. 4c, d). Diabetic rats

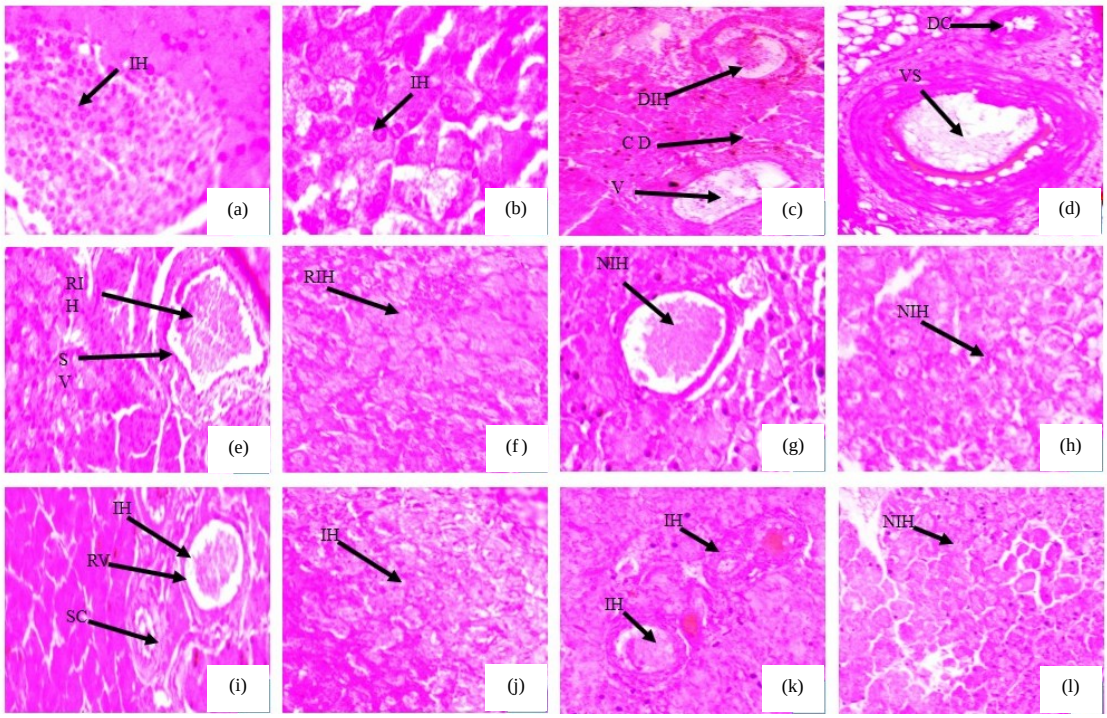

Fig. 4(a-l): Photomicrographs of rat pancreatic sections in the different experimental groups stained with Hematoxylin and Eosin (X100, 400). (a-b) Rat pancreatic sections in control group, (c-d) Pancreas of rat treated with STZ (15 mg kg^{-1}), (e-f) Pancreas of rat treated with Mel (1 mg kg^{-1}) after STZ intoxication, (g-h) Pancreas of rat treated with melatonin alone (*per se*) (1 mg kg^{-1}), (i-j) Pancreas treated with GB after STZ and (k-l) Pancreas treated with GB alone. V: Vacuolation, VS: Vacuole size, DIH: Degenerated islets of langerhans, CD: Cells distracted, SV: Small vacuole size, NIH: Langerhans, IH: Langerhans and RV: Reduce vacuole size

Table 1: Protective effect of exogenous melatonin against STZ induced alterations in pancreatic stress markers and total protein content

Treatments	TBARS (LPO) (nmol mg^{-1} of protein)	GSH (µg mg^{-1} of protein)	SOD (units min^{-1} mg^{-1} of protein)	CAT (units min^{-1} mg^{-1} of protein)	Protein (µg mL^{-1})
CONT	2.60±0.27	29.56±1.67	78.00±4.03	45.750±1.89	16.90±4.35
STZ	5.17±0.64$^{\omega}$	13.12±1.29$^{\omega}$	49.75±3.12$^{\omega}$	28.875±1.29$^{\omega}$	8.69±0.57$^{\omega}$
STZ+MEL	3.20±0.32$^{\#}$	25.46±3.32$^{\#}$	67.75±2.29$^{\#}$	40.475±1.34$^{\#}$	14.50±1.56$^{\#}$
Protection (%)	76.56	75.00	63.71	68.72	70.76
MEL	2.098±0.42	27.57±4.66	75.50±4.34	45.20±2.16	15.86±1.82
STZ+GB	3.670±1.08^{+}	22.10±2.96^{+}	64.10±3.37^{+}	38.70±1.17^{+}	13.20±0.83^{+}
Protection (%)	56.00	54.00	50.65	58.23	54.93
GB	1.95±0.06	23.72±1.02	76.75±4.03	43.21±1.80	14.01±1.95
F-value (at 5% level)	6.035$^{@}$	4.338$^{@}$	8.982$^{@}$	10.820$^{@}$	1.654$^{@}$

Data are Mean±SE, N = 6. CONT: Control, STZ: Streptozotocin, MEL: Melatonin, GB: Glibenclamide, TBARS: Thiobarbituric acid reactive species, GSH: Reduced glutathione, SOD: Superoxide dismutase, CAT: Catalase. $^{@}$Significant at 5% for ANOVA, $^{\omega}$STZ vs. CONT, $^{\#}$STZ vs. STZ+MEL at $p \leq 0.05$, $^{+}$STZ vs. STZ+GB at $p \leq 0.05$

Table 2: Protective effect of exogenous melatonin against the STZ induced decrement of C-peptide

Treatments	C-peptide (ng mL^{-1})	Protection (%)
CONT	0.058±0.004	
STZ	0.027±0.009^{a}	
STZ+MEL	0.046±0.0012^{b}	61.29
MEL	0.570±0.003	
STZ+GB	0.043±0.006^{c}	51.61
GB	0.054±0.0014	

Data are Mean±SEM, N = 6. CONT: Control, STZ: Streptozotocin, MEL: Melatonin, GB: Glibenclamide. F-value = 6.14, $^{@}$Significant at 5% for ANOVA, ^{a}STZ vs. CONT, ^{b}STZ vs. STZ+MEL at $p \leq 0.05$, ^{c}STZ vs. STZ+GB at $p \leq 0.05$

Table 3: Histological alteration in pancreas

Histological remark	CONT	STZ	STZ+MEL	MEL	STZ+GB	GB
Islet cell damage	-	+++	-	-	-	-
Vacuole formation	-	+++	-	-	-	-
Degeneration	-	+++	-	-	-	-
Distortion in cells	-	+++	-	-	-	-
Vacuole size	-	+++	-	-	++	-

-: No damage, +: Slight damage, ++: Moderate damage, +++: Severe damage

treated with exogenous melatonin at a dose of 1 mg kg^{-1} showed recovery with remarkable regeneration of islets of langerhans, reduced vacuole size, well organized cellular architecture. Exogenous melatonin treated photomicrographs were compared with glibenclamide (Standard hypoglycaemic drug) treated group of rats (Fig. 4e, f). Pancreas of rat treated with melatonin alone (*per se*) (1 mg kg^{-1}) did not show any histological impediment (Fig. 4g, h). Pancreas treated with GB after STZ induced intoxication shows newly regenerated islets of langerhans (NIH), small vacuole size (SV), reduce vacuole size (RV) and restoration of histoarchitecture of pancreas (Fig. 4i, j). Pancreas treated with GB alone revealed well maintained cellularity of pancreas, normal islets of langerhans (IH) (Fig. 4k, l). So histopathological observations showed that STZ caused severe pancreatic damage in diabetic rats and exogenous melatonin treatment recovered that damage and melatonin treat rats did not show any damage (Table 3).

DISCUSSION

In the current study assessment of blood glucose level was documented weekly in individual experimental groups as well as mean difference between different groups. Weekly blood glucose level during experimental period showed significant increase in blood glucose level in STZ induced diabetic rats. However, exogenous melatonin treatment significantly restored of glucose level in blood near to the control range. The variations in blood glucose was also calculated between different experimental groups which also confirmed and supported the current results that STZ infact interferes glucose metabolism by degrading insulin produce beta cells and causing hyperglycemic condition. The findings of the present study coincide with previous studies[21].

The STZ induced toxicity causes various biochemical abnormalities and due to the cellular membrane disintegration intracellular components are leaked in the circulation. The STZ toxicity causes reduction in the activities of these anti-oxidant enzymes[21,27]. Melatonin has ability to distribute widely in organisms and cells are believed to be significant feature of its efficacy in quelling free radicals and restricted their damage[37]. In the present study significant decrease was found in GSH, SOD and CAT activities of STZ induced diabetic rats, while as melatonin treatment given to diabetic rats showed significant increase in the activities of these enzymes. Melatonin treated rats in comparison to STZ induced diabetic rats showed significant decrease in lipid peroxidation and ROS generation, which suggested that melatonin prevents the organ damage by protecting lipid from peroxidation by ROS under STZ toxicity[38,39]. Because is melatonin widely used as potent antioxidative molecule, which has both direct antioxidant protective role[40,41] and also indirect antioxidant in the form of N^1-acetyle-N^2-formyl-5-methoxykynuramine (AFMK)[42]. The results were compared with glibenclamide (standard hypoglycaemic drug). The

potential ability of melatonin in the present study to inhibit the oxidative stress mediated ROS production coincides with findings of the studies[22,43].

The C-peptide a key component that plays important role in connecting the A and B chains of the proinsulin molecule, after its cleavage, it is released from pancreatic beta cells in equal molar amounts to insulin[44,45]. The C-peptide is clinically used as a surrogate marker of insulin release and a measure of beta-cell activity in diabetes mellitus[45,46]. Diabetic rats showed significant decrease in C-peptide level, however, melatonin treatment given to diabetic showed significant increase the C-peptide level near to control group. Different studies suggested that C-peptide infusion recovers complications occurring due to diabetes induced by STZ in rats such as albuminuria, proteinuria, glomerular hyperfiltration and hypertrophy in streptozotocin induced by STZ-induced diabetic rats[47-53].

Recent studies reported that volume size of pancreas islets, intensity of cell nucleus as well as cytoplasm richness can displays the function of islet cells, the predominant synthesis of insulin reflects islet regeneration, the more and richer cytoplasm[54]. Diabetic mice showed remarkable deterioration and atrophy, with decreased cytoplasm, cells shrunk and nucleus becomes dense, however, edema and vacuole formation[55]. Melatonin treatment for 4 weeks restored histoarchitecture damages such as restoration of cytoplasmic richness, augmented cell body, decrease in vacuole size and regeneration of islet of cells. Upgraded enzymatic biochemistry variables and histopathological studies also showed recovered structural and functional integrity of the cellular organelles and provided additional support to the proposed protective mechanism of action by melatonin. Melatonin *per se* regulates the biochemical parameters and histological cellular architecture, exhibits non-toxic effect of melatonin.

CONCLUSION

From the findings of the present study, it might be concluded that streptozotocin is associated with oxidative stress in pancreatic tissues. Nonetheless, melatonin exhibited antioxidant activity and has potential to reduce and /or prevent pancreatic oxidative damage generated by streptozotocin.

SIGNIFICANCE STATEMENT

This study discovered the protective role of exogenous melatonin against the diabetes induced pancreatic damages that can be beneficial to combat complication in the other associated organs and their coordinated function. This study will help the researcher to unfold the critical areas of research by tracing the molecular mechanism of melatonin action at receptor as well as non-receptor mediated level that many researchers were not able to explore. Thus a new theory on melatonin a promising natural molecule may be a new scientific approach.

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