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Melatonin and insulin modulates the cellular biochemistry, histoarchitecture and receptor expression during hepatic injury in diabetic rats

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ABSTRACT

Aims; The present study was designed to ameliorate the integrated efficacy of exogenous melatonin and insulin on tissue biochemical, serological, histopathological architecture and receptor expression of melatonin (MT1, MT2) and insulin receptor (IR) expression against the hepatic injury in diabetic rats. Materials and Method; the rats were randomly allocated into nine different experimental groups. Diabetes was induced by streptozotocin (15 mg/kg) for 6 days. Rats having blood glucose level above 250 mg/dl were considered as diabetic. Animals euthanized after 4 weeks, blood and liver samples were collected to perform various biochemical, serological, histopathological and receptor expression of melatonin (MT1, MT2) and insulin receptor (IR). Key findings; Diabetic rats revealed significant increase in lipid peroxidation (LPO) of liver tissue, liver function tests (ALT, AST and ALP), increase in serum cholesterol, LDL, VLD, but decrease in HDL level. Further, diabetic rats exhibited significant decrement in antioxidative enzymatic system (GSH, SOD, CAT, GR, GPX, G6PDH and GST), total tissue protein and glycogen content. Histomicrograph of liver of diabetic rats resulted in vacuolization indicating cellular damages as well as upregulation in liver MT1, MT2 and IR protein expression. However, the combined therapy (Melatonin and insulin treatment) revealed significant recovery and restoration in biochemical, cellular architecture of liver cells and receptor expression pattern of MT1, MT2 and IR. Significance; It may establish a synergistic action of melatonin and insulin, which might be a novel evidence for clinicians to combat the hepatic complication along with controlling diabetes.

1. Introduction

Diabetes is a serious, common, expensive, yet manageable disease. It is a main public health problem and is a major cause of morbidity and mortality [1]. The high prevalence of this disease is due to rapid urbanization, sedentary life style, unhealthy diet and increasing life expectancy [1]. It causes economic burden at individual as well as at family level and raise the cost of care for the health system. Its incidence has greatly increased in the last years but with a major ignorance of its other complication. Diabetes causes several complications that may cause blindness, kidney failure, loss of limbs, hepatic damages, neuronal impairments, immune compromistion. Hepatic damage is one of the major problems in diabetic patients, but still least attenuation has been given regarding this health issue. Liver is the most complex vital organ, involved in many biochemical or metabolic pathways which are involved to fight against diseases, transport nutrients and carbohydrate, lipid and protein metabolism. Therefore, considering its role in different functions, it malfunctioning may cause different complications. Different medicines are available which can control diabetes and complications, but yet the problem remained unsolved. Therefore, an attempt was made to control the diabetes and hepatic injury by overcoming the deficiency of internally produced chemical signaling molecules. Present study hypothesized that exogenous supplementation of melatonin and insulin together might have potential to protective the hepatic damages.

Melatonin (N-acetyl-5-methoxytryptamine) is synthesized in the pineal gland, acting as a neuroendocrine transducer, regulating the day/night cycle. Melatonin regulates physiological synchronization of glucose metabolism and stimulates insulin secretion (GSIS) as well. It mediates various signaling pathways in pancreatic islets through two membrane receptors (MT1, MT2). Being lipophilic nature melatonin diffuses easily through the biomembranes, and nucleus. Previous studies reported that melatonin is powerful antioxidant in biological systems as well as acts as immune regulator [2,3]. It scavenges the reactive

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oxygen and nitrogen species (ROS and RNS respectively) [4]. Melatonin also inhibits some prooxidant enzymes, such as NADPH oxidase [5] and activates the expression pattern of antioxidant enzymes (GPX, GR, G6PDH, CAT and SOD). It also increases the level of reduced glutathione (GSH) by neutralizing the free radical being an electron rich molecule [6,7]. Insulin a peptide hormone is essential for the carbohydrate homeostasis, growth and development of tissues. It stimulates transportation of glucose into the peripheral tissues such as muscles and adipocytes, but inhibits the gluconeogenesis and glycogenolysis in liver.

Previous studies described that melatonin has inhibitory action on insulin secretion through receptor mediated pathway (MT1, MT2) [8]. However, melatonin can acts via cAMP pathway to stimulate the insulin secretion. Present study aims to elucidate the impact of exogenous melatonin and insulin alone and in combination against the diabetes induced hepatic injury.

2. Materials and methods

2.1. Animal maintenance

Healthy albino Wistar strain male rats of same age weighing approximately 190 ± 10 gm were procured from Defence Research and Development Establishment (DRDE) Gwalior, M.P. India. Animals were acclimatized under standard husbandry conditions [(25 ± 2) ^{0}C temp, 60%–70% relative humidity, 14 h light and 10 h dark]. All experimental procedures were performed in accordance with the national and international guidelines and regulations and were approved by SLT institute of Pharmaceutical Sciences (institutional practices of Institutional Animal Ethics Committee) (IAEC), Guru Ghasidas Vishwavidayalaya, Bilaspur Institutional Animal Ethics Committee (Registration Number: 994/Go/ERe/S/06/CPCSEA) (Reference No. 157/IAEC/Pharmacy/2016). Streptozotocin and melatonin was procured from Sigma Aldrich, insulin from Insulin injection was purchased from Actrapid, Novo Nordisk, A/S-Denmark. Egyptian Drug Trading Company, Glibenclamide, from Ahmadabad, Gujrat, India.

2.2. Induction and confirmation of diabetes

The diabetic conditions in male rats were induced by intraperitoneal administration of streptozotocin 15 mg/kg for six days. Blood glucose level was observed by monitoring the changes in the blood glucose following the injection of streptozotocin. Animals with blood glucose level above 250 mg/dl were considered as diabetic (Table 2; Fig. 1). Estimation of random sugar level in serum was also estimated using kit (Table 2). After confirmation of induction of diabetes, animals were divided into nine groups under the following experimental design for four weeks (Table 1).

At the end of experiment (4weeks), animals of each group were euthanized and sacrificed. Liver was dissected out and were fixed in Bouin's fluid for histological observations. Liver were processed for LPO, GSH, SOD, CAT and GSH cycle (GPX, GR & G6PDH) and GST estimation. Serum was separated from the blood by centrifugation at 3000*g* and was stored as 20 °C for serological analysis. Serum was examined for ALT, AST, ALP, total serum protein content (albumin and globulin) and lipid profile (TG, CHOL, LDL, VLDL and HDL), melatonin (MEL) and insulin (INS).

2.3. Evaluation of oxidative stress markers and antioxidant status

Lipid peroxidation in the liver samples was assessed by following the method of Ohkawa et al., 1979 [9]. Free radical load in the liver samples was assessed by the formation of pink colored complex thiobarbutaric acid reactive oxygen species (TBARS) between Malonaldehyde and thiobarbutric acid was evaluated Reduced Glutathione (GSH) was quantified by its reaction with 5-5′-dithiobis 2-nitrobenzoic acid to produce a yellow colored product following the method of Sedlak and Lindsay, 1968 [10]. Superoxide dismutase activity was determined by centrifuging the whole reaction mixture to separate butanol layer containing chromogen and absorbance was taken at 560 nm by following the method of Kakkar et al., 1983 [11]. Catalase was observed by assessing decomposition of hydrogen peroxide/min by following the method of Beers and Sizer, 1952 [12]. Glucose-6-phosphate dehydrogenase in the liver was analyzed when the extracted enzyme oxidizes Glucose-6-Phosphate to 6-Phosphogluconate and simultaneously reduces co-enzyme NADP to NADPH giving increase in absorbance at 340 nm by following the method of Ells and Kirkman, 1961 [13]. Glutathione peroxidase activity was determined when known amount of liver homogenate preparation were allowed to react with H_2O_2 in presence of GSH for a specific time period by following the method of Paglia and Valentine, 1967 [14]. Glutathione reductase activity was assayed in the liver when enzymatic catalytic activity by following decrease in absorbance at 340 nm due to the oxidation of NADPH by following the method of Goldberg and Spooner, 1983 [15]. Glutathione-S-transferase (GST) was assessed by colorimetric method, when interaction between CDNB and glutathione is completely dependent on active GST presence by following the method of Habig et al., 1974 [16]. Glycogen was quantified in liver by allowing the reaction mixture to cool and then boil and again cooled and reading was recorded at 625 nm by adopting the method of Seifter et al., 1950 [17]. Total protein content in the liver samples was determined, when proteins in the samples react with Folin's Ciocalteau reagents to form a colored complex. The compounds react with Folin's reagents, which results aromatic amino acids reduce the phosphomolybdic acid and oxidize them. Formation of blue colour, the intensity of colour is directly proportional to the protein concentrations by following the method of Lowry et al., 1951 [18].

2.4. Evaluation of serum hepatic biochemical markers

Serum was used for the assessment of alanine amino transferase (ALT), aspartate amino transferase (AST), and alkaline phosphatase (ALP), bilirubin, protein content, albumins, globulins, triglycerides, cholesterol, low density lipoproteins (LDL), very low density lipoproteins (VLDL) and high density lipoproteins (HDL) level in serum. Following kits were used ERBA SGPT (ALT) kit (IFCC method), ERBA SGOT(AST)

Table 1
Experimental design.

Group No.	Groups	Treatment	No. of Rats
I	Control (CON)	0.1 M Sodium citrate buffer	6
II	Streptozotocin (STZ)	15 mg/kg body weight, o. p.	6
III	Streptozotocin (STZ) + Melatonin (MEL)	STZ 15 mg/kg (6days) + 1 mg/kg BW (4 weeks)	6
IV	Melatonin (MEL)	1 mg/kg BW (4 weeks)	6
V	Streptozotocin (STZ) + Insulin (INS)	15 mg/kg (6days) + 0.5 ml/kg for 4 weeks	6
VI	Insulin (INS)	0.5 ml (20units)/kg for 4weeks	6
VII	Streptozotocin (STZ) + Melatonin (MEL) + Insulin (INS)	15 mg/kg (6days) + 1 mg/kg + 0.5 ml (20units)/kg (4 weeks)	6
VIII	STZ + Glibenclamide GB	STZ 15 mg/kg + 0.5 mg/kg BW (4weeks) o. p.	6
IX	Glibenclamide (GB)	0.5 mg/kg BW (4 weeks).	6

Table 2
Effect of exogenous melatonin and insulin alone and in combination on STZ induced diabetic rat model with respect to the weekly blood glucose level and serum random sugar level in different experimental groups.

Groups	Weekly changes in blood glucose level (mg/dl)					Random sugar level in serum (mg/dl)
	Before Induction	Week-I	Week-II	Week-III	Week-IV	
CON	125 ± 10	128 ± 8	116 ± 3	120 ± 5	135 ± 4	66.34 ± 3.18
STZ	127 ± 15	301 ± 11**	312 ± 10**	320.33 ± 14***	328 ± 16***	94 ± 4.45*
STZ + MEL	117.25 ± 9	394.75 ± 14**	280.25 ± 15*	243.25 ± 15**	221 ± 18**	69 ± 6.90*
MEL	110 ± 7	120 ± 12	131 ± 10	130.5 ± 8	125 ± 14	65.17 ± 3.22*
STZ + INS	118.25 ± 13	394.75 ± 13**	283.25 ± 6*	219.25 ± 16*	167.2 ± 10**	68.67 ± 7.28
INS	110 ± 10	123 ± 15	135 ± 12	132.5 ± 12	130.5 ± 7	65.67 ± 4.36
STZ + MEL + INS	115 ± 7	298 ± 9***	248 ± 20**	232 ± 20***	224 ± 10***	67.84 ± 3.71**
STZ + GB	114.25 ± 5	384.25 ± 11**	274 ± 17**	186.75 ± 13*	180.5 ± 12**	66.5 ± 4.51*
GB	117.75 ± 7	128 ± 16	140.75 ± 11	125.5 ± 17	144.25 ± 5	63.17 ± 4.34

@ANOVA 5.61 (Random sugar level). Data are Mean ± SE; N = 6. Abbreviations: CON = Control; STZ = Streptozotocin; MEL = Melatonin; INS = Insulin GB = Glibenclamide.
@ Significant at 5% for ANOVA. STZ vs CON STZ vs STZ + MEL.STZ vs STZ + MEL + INS.STZ vs STZ + GB Superscripts denotes; *p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001.

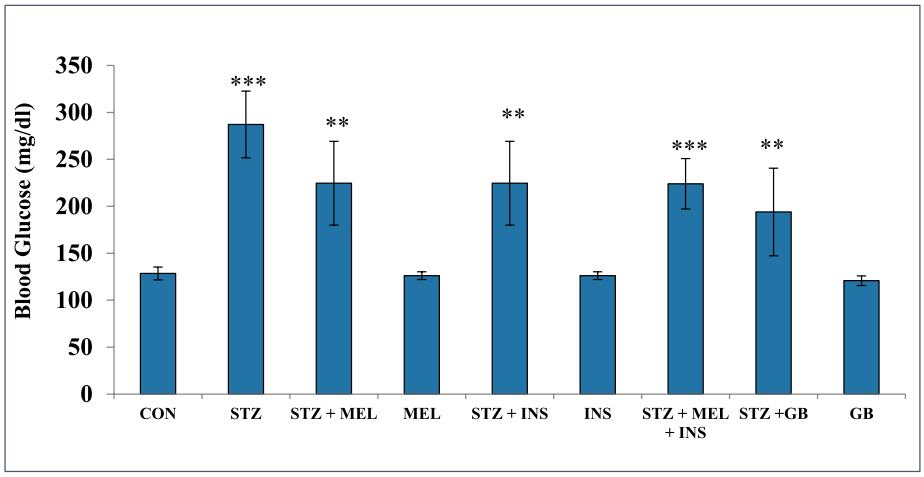

Fig. 1. Effect of exogenous melatonin and insulin on blood glucose level of streptozotocin (STZ) induced diabetic rats. Histogram represent mean ± S.E; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin, GB = Glibenclamide. **p < 0.01 and ***p < 0.001. CON Vs STZ, STZ Vs STZ + MEL, STZ vs STZ + MEL + INS and STZ Vs GB.@ F Value 14.8 @Significance at 5%.

Table 3
Restorative potential of exogenous melatonin and insulin (% protection) on serum biochemical variables against diabetes induced hepatotoxicity.

Groups	ALT(IU/L)	AST (IU/L)	ALP (IU/L)	Bilirubin (mg/dl)
CON	22.33 ± 2.33	17.83 ± 1.67	65.33 ± 2.07	0.45 ± 0.02
STZ	38.17 ± 4.30***	38 ± 2.10***	104.17 ± 2.67***	0.89 ± 0.05***
STZ + MEL	23.93 ± 1.91**	28.5 ± 2.86*	79 ± 3.97**	0.60 ± 0.09**
% Protection	73.43	65.04	64.82	65.90%
MEL	18.17 ± 1.58	23 ± 2.31	56.33 ± 3.23	0.43 ± 0.05
STZ + INS	22.16 ± 3.19**	27.17 ± 2.92**	77.5 ± 3.40**	0.69 ± 0.09**
% Protection	82.67	75.20	68.62745	66.02%
INS	17.5 ± 1.54	21 ± 1.64	56.16 ± 2.12	0.48 ± 0.07
STZ + MEL + INS	18.1 ± 1.72***	23 ± 2.33**	68.5 ± 3.79***	0.46 ± 0.08***
% Protection	100	100	86.71	97.72%
STZ + GB	24.17 ± 1.56**	27.33 ± 2.48**	74.83 ± 4.45**	0.73 ± 0.05*
% Protection	72.18	72.96	75.560133	36.36%
GB	17.67 ± 1.08	22.17 ± 2.08	66.33 ± 4.47	0.42 ± 0.09
ANOVA	**9.773**	**6.317**	**21.356**	26.93

Data are mean ± S.E; N = 6.
Abbreviations: CON = Control; STZ = Streptozotocin; MEL = Melatonin; INS = Insulin; GB = Glibenclamide; ALT = Alanine aminotransferase, AST = Aspartate aminotransferase; ALP = Alkaline phosphate.
@ Significant at 5% for ANOVA. *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001.
STZ vs CON.
STZ vs STZ + MEL.
STZ vs STZ + INS.
STZ vs STZ + MEL + INS.
STZ vs STZ + GB.

Table 4
Restorative potential of exogenous melatonin and insulin (% protection) on blood biochemical variables against diabetes induced hepatotoxicity.

Groups	TG (mg/dl)	CHOL (mg/dl)	LDL (mg/dl)	VLDL (mg/dl)	HDL (mg/dl)
CON	77.5 ± 5.1	122.32 ± 5.96	66.5 ± 3.25	14.11 ± 1.01	72.33 ± 4.89
STZ	102.2 ± 4.69**	155 ± 3.84**	96.83 ± 5.7***	23.28 ± 1.99***	40.5 ± 5.42**
STZ + MEL	85.4 ± 5.28*	143 ± 1.83*	72.5 ± 4.52**	20.16 ± 2.28**	60 ± 6.54*
% Protection	68.01	36.71	80.217606	34.023991	61.262959
MEL	76.5 ± 5.10	123.5 ± 2.51	67.53 ± 6.62	12.22 ± 1.99	70.16
STZ + INS	82.17 ± 7.45**	137.83 ± 3.29*	77.83 ± 4.02*	17.72 ± 1.32**	64.16 ± 6.21*
% Protection	81.09	43.28	62.64424	60.632497	74.33239
INS	74.5 ± 3.21	123.6 ± 1.88**	65.93 ± 4.26	14.73 ± 1.63	70.5 ± 4.20
STZ + MEL + INS	82.83 ± 7.21**	125.33 ± 3.05	67 ± 2.52**	15.26 ± 1.44**	66.83 ± 2.52**
% Protection	78.43	90.78	98.35	87.459105	82.720703
STZ + GB	82.82 ± 5.51*	138.33 ± 5.45*	74.5 ± 4.23*	14.73 ± 1.68**	59.67 ± 2.96**
% Protection	78.46	51	73.62347	93.238822	60.22620
GB	73.5 ± 6.94	124.66 ± 3.81	68 ± 3.36	15.05 ± 0.89	67.2 ± 7.01
ANOVA	**2.272**	**11.197**	**5.958**	**3.940**	**3.02**

Data are mean ± S.E; N = 6.
Abbreviations: CON = Control; STZ = Streptozotocin; MEL = Melatonin; INS = Insulin; GB = Glibenclamide; TG = Triglyceride; CHOL = Cholesterol; LDL = Low Density Lipoprotein; VLDL = Very Low Density Lipoprotein and HDL = High Density Lipoprotein.
@ Significant at 5% for ANOVA. *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001.
CON vs STZ.
STZ vs STZ + MEL.
STZ vs STZ + INS.
STZ vs STZ + MEL + INS.
STZ vs STZ + GB.

Table 5
Protective therapeutic potential of exogenous melatonin and insulin (% protection) on total protein content, albumin and globulin against diabetes induced hepatotoxicity.

Groups	Total serum protein content (g/dl)	Albumin (g/dl)	Globulin (g/dl)
CON	8.58 ± 0.75	4.59 ± 0.18	4.21 ± 0.46
STZ	3.75 ± 0.26*	2.55 ± 0.32***	1.55 ± 0.30**
STZ + MEL	7.44 ± 0.59**	3.91 ± 0.29**	3.55 ± 0.35*
% Protection	76.39%	66.66%	75.18%
MEL	7.95 ± 0.81	24.08 ± 0.33	3.92 ± 0.52
STZ + INS	7.43 ± 0.41**	3.78 ± 0.27**	3.62 ± 0.34*
% Protection	76.39%	63.01%	75.02%
INS	7.62 ± 0.55	4.08 ± 0.33	3.83 ± 0.32
STZ + MEL + INS	8.05 ± 0.47***	4.14 ± 0.26***	3.95 ± 0.16***
% Protection	89.02%	77.94%	90.22%
STZ + GB	7.37 ± 0.59**	3.92 ± 0.25**	3.58 ± 0.49*
% Protection	74.94%	67.15%	76.31%
GB	7.49 ± 0.65	4.01 ± 0.33	3.51 ± 0.33
ANOVA	6.71	5.93	4.86

Data are mean ± S.E; N = 6.
Abbreviations: CON = Control; STZ = Streptozotocin; MEL = Melatonin; INS = Insulin; GB = Glibenclamide.
@ Significant at 5% for ANOVA. *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001.
CON vs STZ.
STZ vs STZ + MEL.
STZ vs STZ + INS.
STZ vs STZ + MEL + INS.
STZ vs STZ + GB.

kit (IFCC method), ERBA ALP kit (IFCC method), ERBA Bilirubin kit (Diazo method), Total protein kit (Biuret method), ERBA Albumin kit (BCG Dye method), ERBA Chol Kit (CHOD-PAP method), ERBA Triglyceride DES kit (GPO Trinder method), ERBA LDL-Cholesterol (Phosphotungstic acid method), VLDL-Cholesterol kit (Phosphotungstic acid method), ERBA HDL-Cholesterol (Phosphotungstic acid method) according to the instructions provided in the manual of commercial kits. All the analytic kits were purchased from ERBA diagnostics Mannheim GmbH Mallaustr, Germany.

2.5. Hormonal assessment

Serum samples were further analyzed for melatonin and insulin using the analytic ELISA kits for measurement of circulatory level of melatonin and insulin. Melatonin (MEL) ELISA kit was purchased from IBL-Germany RES54041) and Insulin ELISA kit was purchased from Actrapid Novo Nordisk, A/S-Denmark. Egyptian Drug Trading Company.

2.6. Histological observations

The liver tissue was washed in normal saline and fixed in Bouin's fluid. Liver samples of all experimental groups were dehydrated using different graded series of ethanol. Samples were cleared using xylene and embedded in paraffin wax. Liver sections of 4–5 μm thick were cut using rotary microtome (Leica RM 2125-RT 5) stained with hematoxylin and eosin (H and E), mounted with DPX and observed under light microscope (Magnus, India, using 10× and 40×).

2.7. Receptor assay

2.7.1. qRT-PCR-iScript™ first standard cDNA synthesis kit (BIORAD)

qRT-PCR was done for the expression assay of melatonin receptors (MT1, MT2) and insulin receptor (IR) by first extracting the total mRNA, followed by cDNA synthesis. The primers were purchased from Imperial life sciences (P) limited.

List of primers

Gene Product	Forward	Reverse
MT1	5′-CGTTGGTGCTGATGTCG-3′	5′-AGTTTGGGTTTGCGGTC-3′
MT2	5′-CAATGCTGCGAGGCG-3′	5′-GGCGGTGGTGACGATG-3′
IR	5′-TCAGAACCCGATGACCCTAC-3′	5′-GGGATGCACTTGTTGTTGTG-3′
β-actin	5′-GGAAATAGGGGTTAGCAC-3′	5′- CTCATGTGCGCCTACTTA-3′

2.7.2. RNA isolation

Total RNA was isolated from the frozen tissue samples. Tissue was slowly crushed with the help of mortar and pestle. 1 ml of RIBOZOL (trizole) was

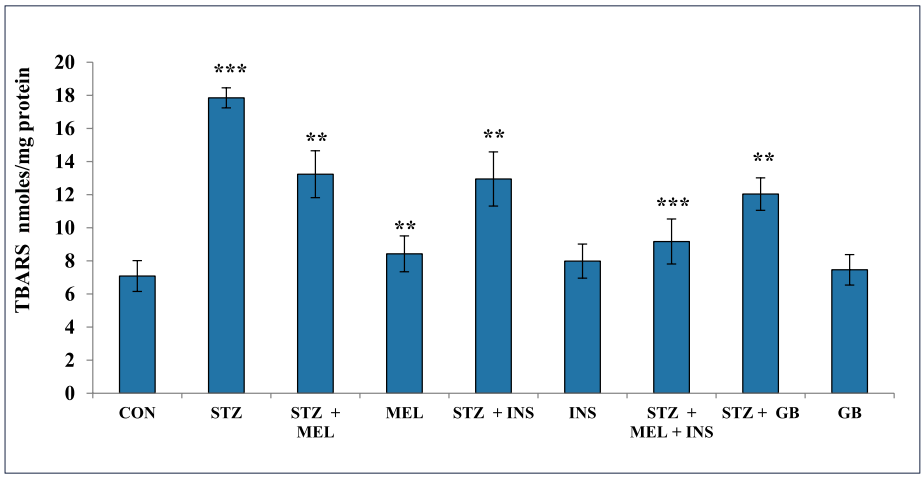

Fig. 2. Effect of exogenous melatonin and insulin on lipid per oxidation (LPO) in liver of STZ induced diabetic rats. Histogram represents mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin and GB = Glibenclamide.**p < 0.01 and ***p < 0.001. CON Vs STZ, STZ Vs STZ + MEL, STZ vs STZ + MEL + INS; STZ Vs GB. @F Value 7.866. @ Significant at 5% at ANOVA.

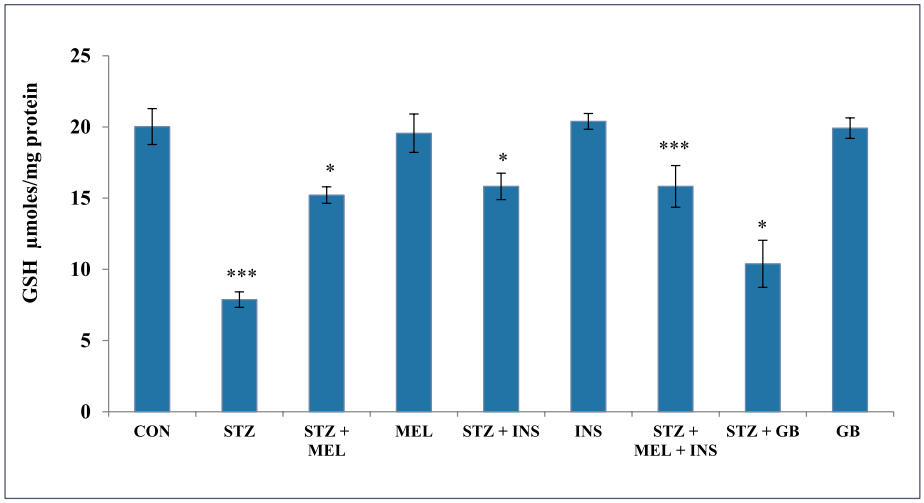

Fig. 3. Effect of exogenous melatonin and insulin on reduced glutathione (GSH) level in the liver of streptozotocin induced diabetic rats. Histogram represents mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin and GB = Glibenclamide. *p < 0.05 and ***p < 0.05.CON vs STZ, STZ vs STZ + MEL, STZ vs STZ + MEL + INS and STZ vs STZ + GB. @ F Value 31.603 @ Significant at 5% at ANOVA.

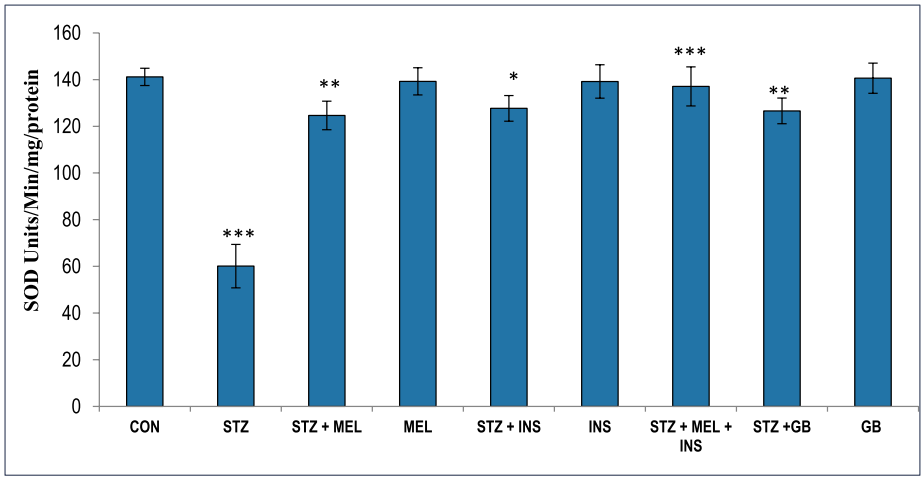

Fig. 4. Effect of exogenous melatonin and insulin on superoxide dismutase (SOD) activity in liver of streptozotocin induced diabetic rats. Histogram represents mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin; GB = Glibenclamide.*p < 0.05; **p < 0.01 and ***p < 0.01.CON Vs STZ, STZ Vs STZ + MEL, STZ vs STZ + MEL + INS and STZ Vs GB.@FValue 17.568. @ Significant at 5% at ANOVA.

added into it and homogenized for 5 min. The tissue samples homogenized in RIBOZOL and transferred to a fresh microtube and left for 5 min at room temperature. Pellet was washed air dried and dissolved in 50 µl of DEPC water and 5 separate aliquots were prepared and kept in 80 °C for further processing. cDNA synthesis was done by using Thermo kit K1632.

2.8. Statistical analysis

Results were expressed as the mean ± SE of six animals used in each group. SPSS (IBM 20.0 version software) comparison between experimental groups was carried out by using One-way analysis of variance (ANOVA) followed by student's t-test computed at $p \leq 0.05$ [19].

3. Results

3.1. Assessment of hepatic injury

Due to uncontrolled metabolism of glucose, the excess glucose gets

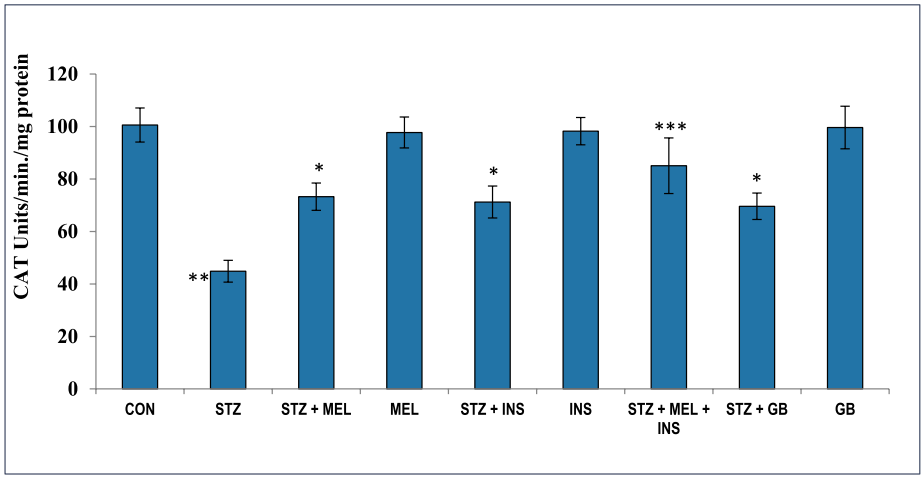

Fig. 5. Effect of exogenous melatonin and insulin on catalase (CAT) activity in liver tissues of streptozotocin induced diabetic rats. Histogram represents mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = insulin and GB = Glibenclamide.*p < 0.05; **p < 0.01 and ***p < 0.01 CON Vs STZ, STZ Vs STZ + Mel, STZ vs STZ + MEL + INS; STZ Vs GB. @F Value 8.733 @ Significant at 5% for ANOVA.

Table 6
Therapeutic influence of exogenous melatonin and insulin (% protection) on LPO, GSH, SOD, CAT, total protein content in and glycogen level in liver against diabetes induced hepatotoxicity.

Groups	Parameters					
	LPO	GSH	SOD	CAT	Protein content (Tissue)	Glycogen
STZ vs STZ + MEL	**79.03%**	**72.21%**	**79.58%**	**51.03%**	**52.85%**	**70.72**
STZ vs STZ + INS	47.50%	76.57%	63.37%	47.36%	66.42%	74.66
STZ vs STZ + MEL + INS	95.17%	65.71%	74.37%	91.34%	86.43%	88.85
STZ vs STZ + GB	79.19%	61.42%	62.01%	44.44%	63.12%	76.39

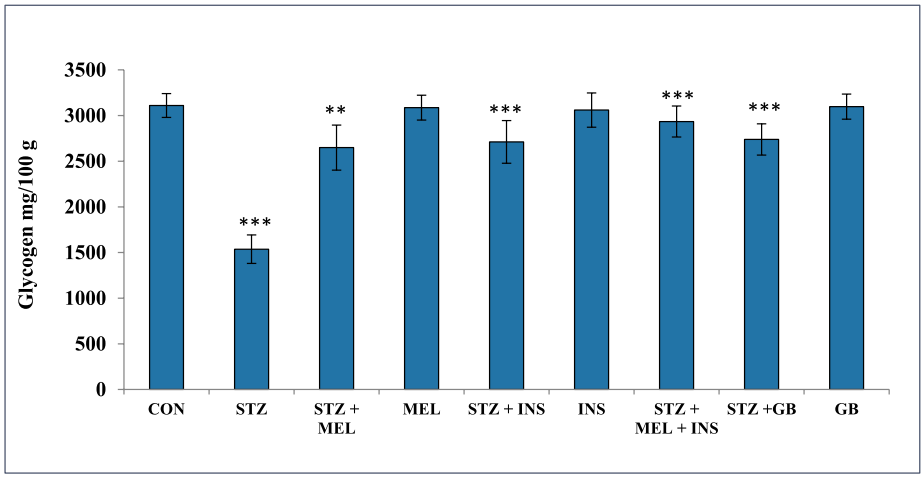

Fig. 6. Effect of exogenous melatonin and insulin on glycogen content in liver in streptozotocin induced diabetic rats. Histogram represents mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin and GB = Glibenclamide. **p < 0.01 and ***p < 0.001. CON Vs STZ, STZ Vs STZ + MEL, STZ Vs STZ + MEL + INS and STZ Vs STZ + GB. @F Value 9.26@ Significant at 5% for ANOVA.

autooxidised and causes various adverse effects on the hepatic cellular biochemistry. Diabetic group of rats revealed remarkable elevation in blood glucose as well as serum sugar level (Fig. 1 and Table 2). This is also the confirmation of induction of diabetes. Significant increase in the hepatic marker enzymes ALT, AST, ALP, bilirubin, triglyceride, cholesterol, low density lipoproteins, very low density low lipoproteins while as significant decrement in high density lipoprotein total serum proteins (albumin and globulins) and level in diabetic animals (Tables 3–5). Alteration in all these biochemical variables revealed hepatic injury during diabetes.

3.2. Assessment of integrated efficacy of exogenous melatonin and insulin against the diabetes induced hepatic injury

Therapeutic efficacy of exogenous supplementation of melatonin and insulin was evaluated against the glucotoxicity induced hepatic injury considering various biochemical fundamental parameters, histopathological and molecular approach to assess the functional as well as structural changes in liver during diabetes.

3.3. Liver specific markers and biochemical variables in serum

Diabetes induced glucotoxic resulted in hepatic injury as revealed by significant augmentation in liver specific markers ALT, AST, ALP, bilirubin, triglyceride, cholesterol, LDL, VLDL along with decrement in HDL, protein content (albumin and globulin) in comparison to control group (Tables 3–5). Administration of melatonin and insulin showed recovery and restoration in all the variables. However, significantly higher rate of recovery was observed in the group of animals treated with combination of both. The efficacy of melatonin and insulin was equivalent to that of positive antidiabetic drug glibenclamide treated group. Animals treated with only melatonin and insulin does not showed any undesirable result in all the serum variables.

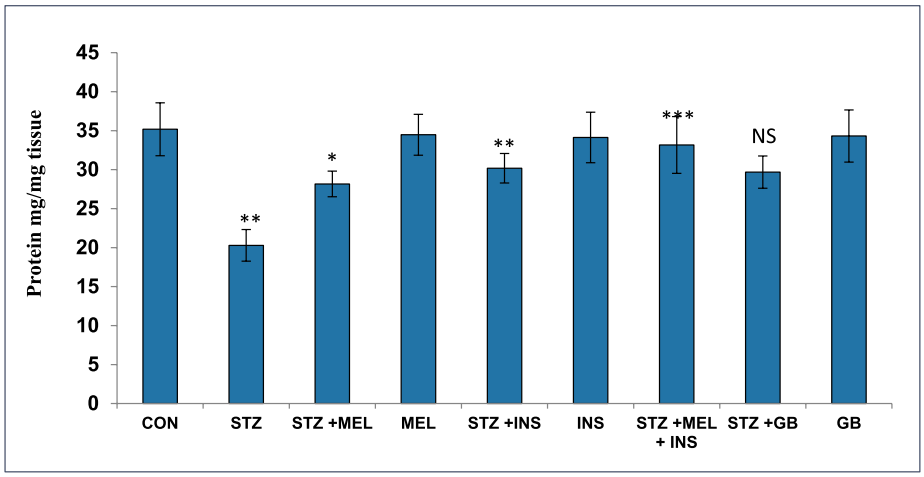

Fig. 7. Effect of exogenous melatonin and insulin on total protein content in hepatic tissues of STZ induced diabetic rats. Histogram represents Mean ± SE; n = 6, CON = Control, STZ = Streptozotocin, MEL = Melatonin, INS = Insulin and GB = Glibenclamide.*p < 0.05, **p < 0.01, ***p < 0.001. CON Vs STZ, STZ Vs STZ + MEL, and STZ vs STZ + MEL + INS; STZ Vs GB.@F Value 5.66 @ Significant at 5% for ANOVA.

Table 7
Protective effect of exogenous melatonin and insulin (% protection) on GSH cycle enzymes (GR, GPX and G6PDH) and GST in liver against diabetes induced hepatic damages.

Groups	GSH cycle enzymes ((Units/min/mg protein)			GST (Units/min/mg protein)
	GR	GPX	G6PDH	
CON	43.6 ± 3.04	7.02 ± 0.48	43.09 ± 2.72	606.65 ± 27.53
STZ	23.77 ± 1.99***	3.25 ± 0.45**	21.07 ± 2.61***	355.7 ± 18.12***
STZ + MEL	38.96 ± 1.92**	6.05 ± 0.38	38.81 ± 1.38**	521.37 ± 41.90**
% Protection	76.60%	74.27%	80.56%	66%
MEL	42.97 ± 1.89	6.69 ± 0.94	47.84 ± 2.19	614.97 ± 16.98**
STZ + INS	39.23 ± 1.68*	6.06 ± 0.49*	37.90 ± 1.46*	511.47 ± 35.29
% Protection	77.96%	74.53%	76.43%	62.07%
INS	42.23 ± 2.12	6.9 ± 0.75	43.19 ± 2.12	598.33 ± 29.32
STZ + MEL + INS	43.08 ± 1.75***	6.87 ± 0.58***	42.78 ± 1.75***	595.19 ± 39.93***
% Protection	97.37%	96.02%	98.59%	95.43%
STZ + GB	41.86 ± 1.96*	5.67 ± 0.56*	36.96 ± 2.92**	499.97 ± 24.29*
% Protection	91.22%	64.19%	72.16%	57.48%
GB	44.48 ± 2.23	6.67 ± 0.89	43.86 ± 2.20	596.03 ± 21.56
@ANOVA	10.91	4.09	12.58	9.54

Data are mean ± S.E; N = 6.
Abbreviations: CON = Control; STZ = Streptozotocin; MEL = Melatonin; INS = Insulin; GB = Glibenclamide; GR = Glutathione Reductase; GPX = Glutathione Peroxidase; G6PDH = Glucose-6-Phosphate Dehydrogenase and GST = Glutathione-S-Transferase.
@ Significant at 5% for ANOVA. *p ≤ 0.05; **p ≤ 0.01 and ***p ≤ 0.001.
CON vs STZ.
STZ vs STZ + MEL.
STZ vs STZ + INS.
STZ vs STZ + MEL + INS.
STZ vs STZ + GB.

3.4. Markers of oxidative stress and tissue biochemical state

The unmetabolized gets oxidized during diabetic condition, which results in the production of free radicals. These free radicals elevate the rate of lipid peroxidation in liver (Fig. 2). Increase in the TBARS level directly indicates the excessive free radical production, elevation in oxidative stress and liver injury. Treatment of melatonin and insulin prevented the free radical, but combined treatment of both the hormones restored the lipid peroxidation rate significantly higher. Reduced glutathione (GSH) (Fig. 3) was diminished in diabetic animals; however treatment of melatonin and insulin increased the GSH level. However, significant increase in GSH level was observed in the group of animals treated with combination of both as that of the control group. Total protein content and glycogen level was decreased in the diabetic animals, animals treated with the alone treatment of melatonin and insulin does not showed significant increase in the total protein and glycogen level. However animals treated with combination of melatonin and insulin significantly increased the level of protein and glycogen near to the control range (Figs. 4 and 5 and Table 6).

3.5. Antioxidant status

Activity of superoxide dismutase (SOD) and catalase (CAT) was significantly declined in the diabetic induced hepatic injury group of animals. Administration of melatonin and insulin recover the decreased activity of SOD and CAT. But integrated treatment of melatonin and insulin showed significantly higher recovery in the both the enzymatic activities. Animals treated only with melatonin and insulin does not reveal any adverse change in SOD and CAT activities, but remains within control range (Figs. 6 and 7). It is very interesting that combination of melatonin and insulin regulate the glucose metabolism, as melatonin is a chrononbiotic as well as antioxidant on the other side insulin is a key regulator of glucose homeostasis. Diabetic animals caused considerable inhibition/suppression of glutathione peroxidase (GPX), glutathione reductase (GR), glucose-6- phosphate dehydrogenase (G6PDH), and glutathione-S-transferase (GST) in liver (Table 7). Administration of melatonin and insulin showed significant restoration in glutathione dependent antioxidative enzymatic activities near to the control level. Results revealed that alone treatment of

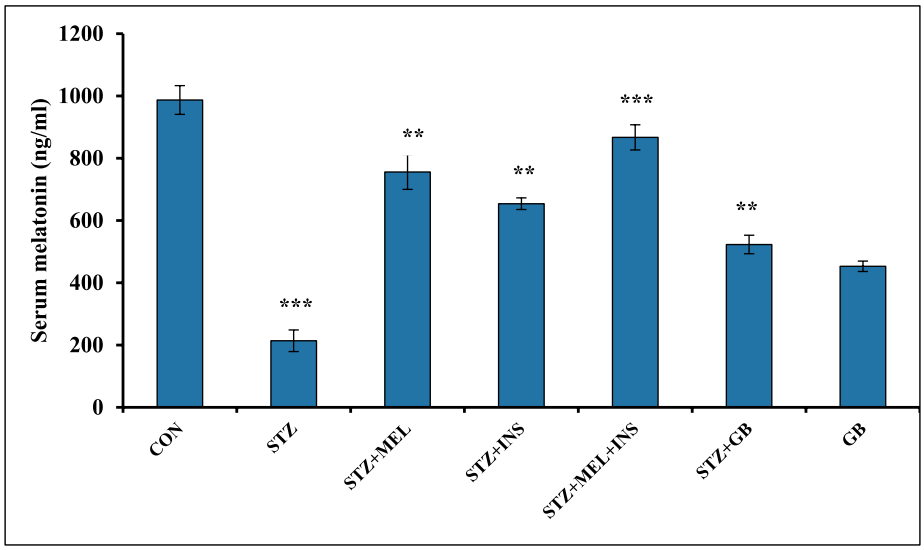

Fig. 8. Combined effect of exogenous melatonin and insulin on serum melatonin level of streptozotocin induced male diabetic rats. Histogram represents Mean + SE; N = 6.CON = Control, STZ = Streptozotocin, MEL = Melatonin, GB = Glibenclamide. *p < 0.05; **p < 0.01; ***p < 0.001; @ Significant at 5% for ANOVA, @F Value 10.56.

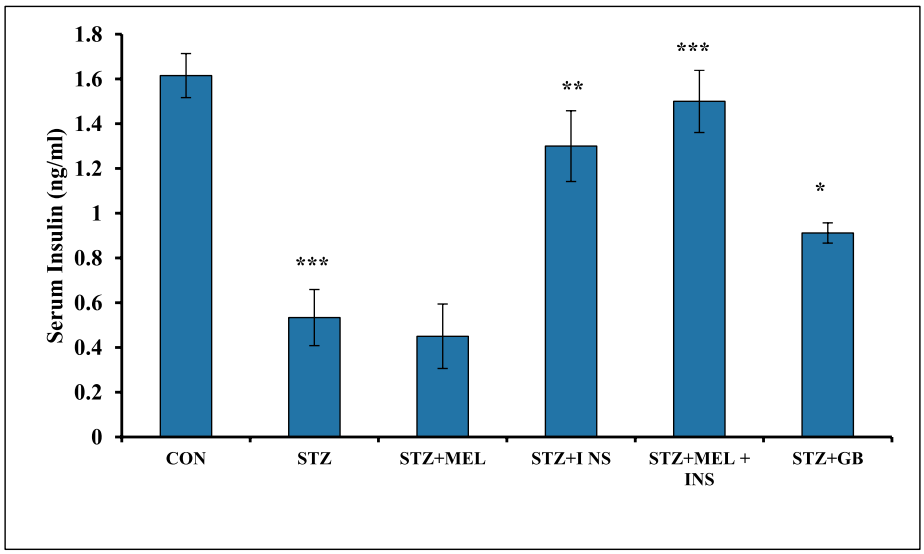

Fig. 9. Effect of combined treatment of melatonin and insulin on serum insulin level of streptozotocin induced male diabetic rats. Histogram represents Mean + SE; N = 6.CON = Control,STZ = Streptozotocin,MEL = Melatonin,GB = Glibenclamide. *p < 0.05; **p < 0.01; ***p < 0.001 @ Significant at 5% for ANOVA. @F Value 8.40. CON vs STZ; STZ vs STZ + MEL; STZ vs STZ + INS; STZ vs STZ + MEL + INS; STZ vs STZ + GB. CON vs STZ; STZ vs STZ + MEL; STZ vs STZ + INS; STZ vs STZ + MEL + INS; STZ vs STZ + GB.

melatonin and insulin given to the animals does not revealed any alteration in these enzyme activities.

3.6. Hormonal assessment

Diabetic rats exhibited significant reduction in serum melatonin and insulin level in comparison to control group. Whereas exogenous supplementation of melatonin and insulin to the diabetic rats for four weeks showed significantly increased the melatonin and insulin near to control and glibenclamide treated groups (Figs. 8 and 9).

3.7. Histopathological examination

Histopathological observations of control group showed normal histoarchitecture of hepatocytes with prominent nucleus, regular arrangement of hepatic cords and conserved sinusoidal spaces diabetic liver showed a variety of cellular artifacts. Diabetic group of animals exhibited deterioration of hepatic cells, irregular and damaged hepatocytes, characterized by hepatic lesions necrosis, vacuolation, indistinct hepatic cords, inflammatory cell infiltration, and obliterated sinusoids. Treatment of melatonin and insulin given to diabetic rats revealed significant regeneration in central vein obstruction, regular lobular arrangement with well-formed polygonal hepatocytes containing conspicuous nucleus, and broader sinusoidal spaces (10X and 40X) (Fig. 10a–b).

From the above mentioned histological observations it can be concluded that polytherepeutic treatment of melatonin and insulin was significantly effective against the diabetes induced hepatic cellular damages. Therefore, a molecular approach was designed to evaluate the administration of melatonin and insulin in the regulation of receptor expression of melatonin (MT1, MT2) and insulin receptor (IR).

3.8. Assessment of relative expression pattern of MT1, MT2 and insulin receptor (IR) in hepatocytes

Expression of MT1 and MT2 receptors showed alteration in expression level in hepatocytes. However, change in expression of MT1 receptor were remarkable stronger than MT2 receptor. Melatonin and insulin treatment alone and the group of rats received combined treatment resulted in significant restoration in the relative expression of

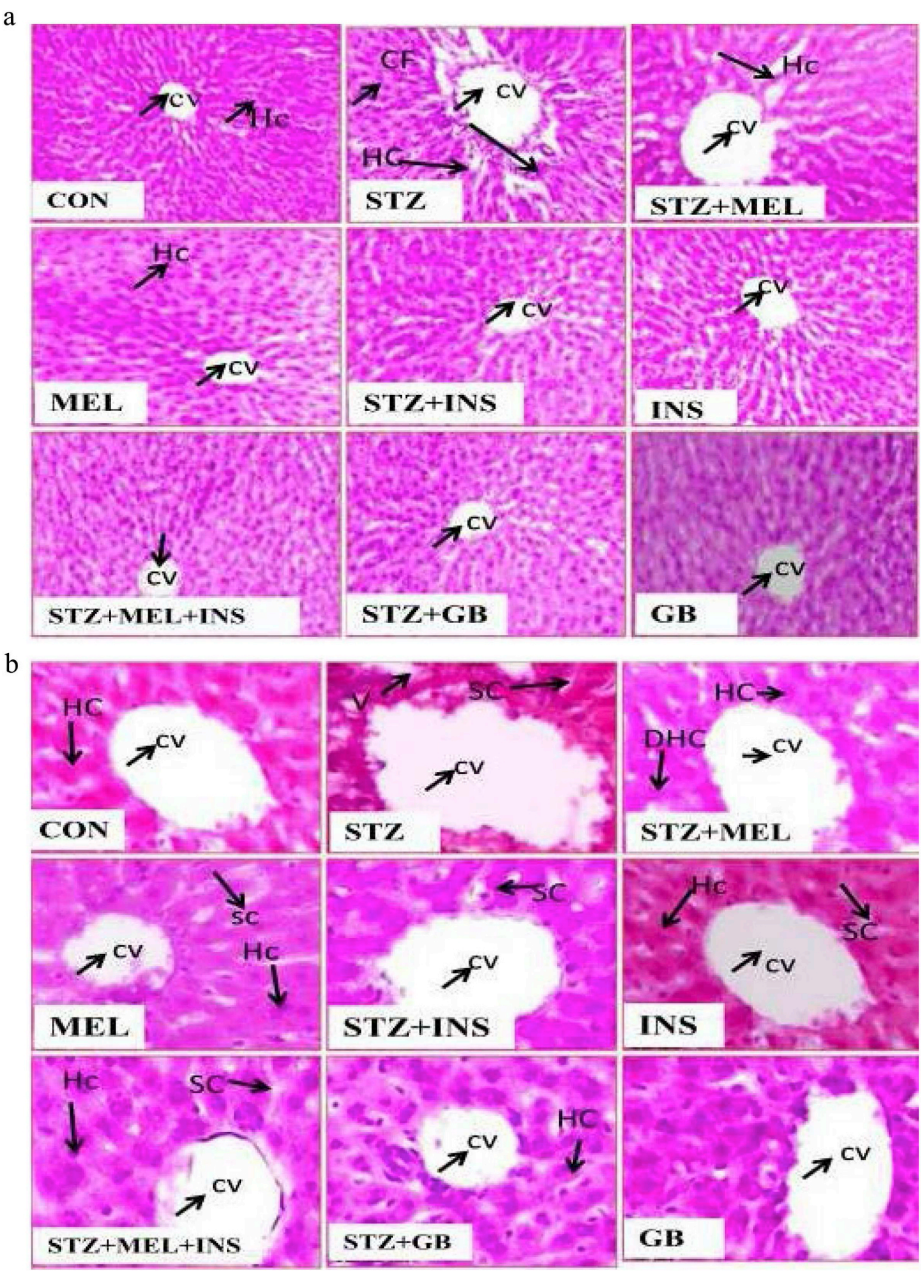

Fig. 10. (a–b): Histomicrograph of liver showing effect of melatonin and insulin alone and in combination in all experimental groups. CON- Control showing normal hepatocytes and well developed central vein (CV), STZ-Streptozotocin treated group showing damaged central vein and accumulation of cell debris in central vein of hepatocytes, vacuolization, necrosis, dilation of sinusoid and distorted hepatic cords. STZ + MEL: Streptozotocin (STZ) + Melatonin (MEL) injection has restored the cellular damages, MEL- Melatonin treatment restored the cellularity of hepatocytes and central vein (CV), STZ + INS: streptozotocin + insulin administration showed recovery in the cellular alterations. INS-Insulin: treatment restored the cellularity of hepatocytes and central vein. STZ + GB-Glibenclamide + Streptozotocin recovered the cell damage and central vein deterioration and GB-Glibenclamide treatment as a antidiabetic drug maintained the normalcy in liver cells. STZ + MEL + INS: streptozotocin + melatonin and insulin showed significantly higher recovery and restoration in hepatic cellular damages. Magnification - 400X.

both MT1 and MT2 receptors comparable to the control as well as antidiabetic glibenclamide treated liver cells (Fig. 11). Diabetic condition resulted in significant alteration in IR expression in hepatocytes. However, melatonin and insulin alone and in combination treated groups resulted in significant recovery in the IR expression in hepatocytes to that of control and glibenclamide treated groups (Fig. 12).

4. Discussion

Hepatic injuries are the severe alarming situation during treatment of diabetes and its associated complications remain still a challenge to medical practitioners. Present study was conducted to evaluate the therapeutic efficiency of exogenous hormonal (melatonin and insulin) treatment during diabetes to overcome the hepatic damages. Melatonin effects the modulation of insulin secretion in the pancreatic islets as well as potentiating positive effect of insulin on norepinephrine-mediated melatonin synthesis in the pineal gland. Therefore, intimate interdependence of both hormones in the modulation of their synthesis is well confirmed [20–22]. Previous findings suggested that melatonin treatment improves the insulin sensitivity by increasing the glucose decay constant [23]. Insulin exerts many pleiotropic functions and plays central role in the regulation of energy metabolism [24]. Reductions in the expression of insulin receptors as well as increase in insulin resistance are two major factors related with ageing [25,26]. Further, it is suggested that melatonin modulates insulin secretion by activating pancreatic islets.

Melatonin and insulin administration significantly decreased the abnormal blood glucose level in diabetic rats. Which can be explained that these two hormones might have synergistic potential to activate beta cells, granulation, hence sensitizes pancreatic beta cells [3]. Melatonin administration enhanced the insulin sensitivity by increasing the glucose decay constant [27].

Findings of the present study evidenced significantly decrease in the body weight of diabetic rats may be caused by uncontrolled loss of glucose [26], increase in muscle wasting and breakdown of fats and proteins [28]. The findings of the present study revealed significant decreases in cellular and serum protein content which is in correlation with weight loss as observed in diabetic rats. Diabetic rats received

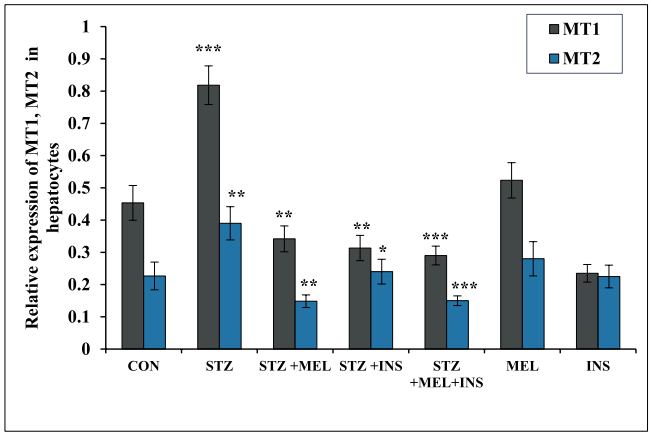

Fig. 11. Effect of exogenous and insulin alone and in combination on relative of IR (Insulin receptor) expression in hepatocytes streptozotocin induced male diabetic rats. Histogram represents Mean + SE; N = 6.CON = Control, STZ = Streptozotocin, INS = Insulin, GB = Glibenclamide.*p < 0.05; **p < 0.01.@ANOVA 21.606@Significant at 5% for ANOVA. CON vs STZ; STZ vs STZ + MEL; STZ vs STZ + INS; STZ vs STZ + MEL + INS; STZ vs STZ + GB.

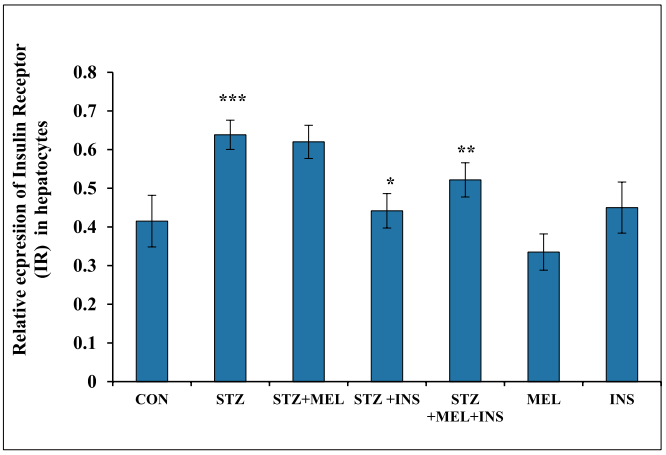

Fig. 12. Effect of exogenous and insulin alone and in combination on relative MT1 and MT2 receptor expression in hepatocytes of streptozotocin induced male diabetic rats. Histogram represents Mean + SE; N = 6.CON = Control, STZ = Streptozotocin, INS = Insulin, GB = Glibenclamide. *p < 0.05; **p < 0.01. @ F Value 21.606 and 19.01, @ Significant at 5% for ANOVA. CON vs STZ; STZ vs STZ + MEL; STZ vs STZ + INS; STZ vs STZ + MEL + INS; STZ vs STZ + GB.

combined treatment of insulin and melatonin for four weeks showed complete restoration in the protein content in the cells and circulatory serum. This in turn prevents the weight loss as well as abnormal glycosylation of proteins. The restoration in cellular and tissue protein content might have occurred because of the inhibitory action of insulin on lipolysis of adipose tissues. Together with the antioxidative function of melatonin preventing oxidative stress induced abnormal protein glycosylation [29].

Diabetic rats showed significant increase in lipid peroxidation; however treatment of combined therapy of melatonin and insulin revealed significant decrease in lipid peroxidation comparable to control group. These results were supported by earlier studies [30] reported that melatonin neutralizes the free radicals. It scavenges ·OH and peroxyl radicals along with insulin mediate its activity by reactivating the glucose metabolism. Hence, these two endocrine hormones might be working together either in additive/synergistic manner to maintain glucose homeostasis. Moreover, melatonin being a highly lipophilic and fairly hydrophobic molecule [31]. Hence, it might works through the non-receptor mediated mechanism path by its unique virtue of free radical scavenging activity. The free radicals generated due to hyperglycemia and/or may diabetic induced, but melatonin and insulin resulted in a significant decrease in blood glucose level. This decrease in blood glucose can be explained on further findings of the present study where diabetic rats showed decreased level of reduced glutathione (GSH) as well as reduced activities the antioxidative enzymatic system such as SOD, CAT and GSH cycle (GPX, G6PDH and GR) and GST enzymatic activities in hepatic system. However, combined treatment of exogenous melatonin and insulin significantly restored the activity of SOD, CAT and GPX, GR, G6PDH, GST and also increased the level of reduced glutathione. The results of the present study coincide with earlier findings [32–34]. GST might be coupled with the melatonin and may act as an enzyme for GSH conjugation reactions [35] lead to the modification in intracellular GSH level of hepatic system in response to ROS generation. Hence can be implicated potency of melatonin in cellular protection against free radical generating agents [36,37]. Thus, the present study suggests that melatonin and insulin together might have reactivated the activities of antioxidative enzymes and reversed all altered enzymatic activities.

Conversion of glucose into glycogen in the hepatocytes depends on the concentration of glucose in the extracellular matrix and also on the insulin availability. Present study revealed that diabetic rats revealed reduced hepatic glycogen content. Administration of insulin and melatonin to diabetic showed significant increase in glycogen content of the liver. Possible reason might be reactivation of the glycogen synthase system. As a consequence of administration of exogenous insulin sensitizes the insulin receptors which inturn initiate the insulin synthesis. The findings are in agreement with earlier reports [38] that glycogen synthetase activity gets inhibited via abnormal glycosylation of enzymes which affect the storage of glycogen in the liver.

Diabetic rats showed significant increase in the hepatocellular marker enzymes such as ALT, AST and ALP. Revealed that diabetes causes glucotoxicity and leads to the disintegration of the hepatocytes due to leakage of these native cellular enzymes into the circulatory blood serum. However treatment of exogenously administered melatonin and insulin might have reduced glucose induced free radical damage in the cellular membranes. The results of this study coincides with previous findings [39–41] reported that diabetes causes damages in the plasma membrane, sinusoidal injury, obstruction of extrahepatic bile flow of hepatocytes.

Hyperbilirubinaemia indicates pathophysiological status of liver. In case of hepatic damage total bilirubin level in serum increase which might be because of the failure of normal uptake, conjugation and excretion by the damaged parenchymatic cells of liver. Diabetic rats showed significantly increased bilirubin level in the serum. However, combined administration of melatonin and insulin restored the bilirubin level near to the control level. Decrease in serum protein content was observed in diabetic rats might be due to microproteinuria. This is an important clinical marker of diabetic nephropathy as well as elevation in the rate of protein catabolism [42]. Whereas, serum protein content showed significant increase after administration of combined treatment of melatonin and insulin to the diabetic rats. It might had occur because of the free radical scavenging activity ability of melatonin as well as regulation of glucose metabolism in hepatic cells and renal cortex, melatonin prevent the adduct formation, while as glucose insulin metabolize the excess glucose and hence prevents its autoxidation and glycosylation of proteins. Circulatory level of melatonin and insulin was found significantly decreased in diabetic rats. However, exogenous melatonin and insulin supplementation significantly upregulated the melatonin and insulin level near to the control. Melatonin augments the PLC/IP3, which activate the release of Ca^{2+} from intracellular stores and this pathway enhances insulin secretion. Therefore, exogenous supplementation of melatonin and insulin could combat the endogenous deficiencies.

Histological observation supports the alterations in biochemical (oxidative stress, antioxidative enzymes, glycogen, total protein

content, lipid profile and liver function enzymes) and hormonal approach. STZ induced diabetic intoxicated animals revealed devastation of architectural pattern, focal necrosis, sinusoidal congestion, focal damage around the central venuole, feathery degeneration, loss of lobular architecture with injured cellular outline and congestion, nuclei have become condensed and pyknotic. The present findings coincides with earlier studies of [43,44] reported that diabetes causes periportal necrosis, cell infiltration and congestion, shrinkage of hepatocytes nuclei and dilation of sinusoid. The animals administered exogenous hormonal therapy (melatonin and insulin) and standard hypoglycemic drug (glibenclamide) showed recovery in histological artifacts such as normal lobular architecture, central and portal veins appear normal. In these experimental groups no necrosis or fatty changes or any inflammatory signs were seen.

Ever since molecular cloning of the MT1-receptor expression studies have indicated a close link between this receptor and inhibitory G-proteins (Gi) and cAMP signaling. Therefore, the molecular mechanism can be determined with an explanation that the effect of melatonin on insulin secretion are mediated through melatonin receptors (MT1 and MT2), which inhibit the AC/cAMP system [45]. In this manner, melatonin reduces the production of cAMP and helps in the release of insulin. Furthermore, melatonin also inhibits the GC/cGMP pathway, possibly via MT2 receptors resulting in the reduced insulin release and hence enhances its level [46]. Previous finding provide evidence that melatonin increases PLC/IP3, which mobilize Ca^{+} from intracellular stores and this pathway increases insulin secretion [47,48].

During pathogenesis of diabetes alterations in the expression of melatonin's own receptor MT1 and MT2 as well as insulin receptor (IR) were evident. Liver of diabetic rats showed significant up regulation suggesting the free availability of melatonin receptors in circulation as the circulatory melatonin was noted among diabetic rats. Elevated expression of MT1 and MT2 receptors were noted in lever cells of male diabetic rats. However insulin injections normalized the expression level to that of control liver cell. The IR expression level of liver cells also corresponds to the MT1 and MT2 receptor expression pattern. The present study clearly deals with the influence of the diabetic metabolic state on melatonin synthesis and secretion of the pineal gland. The findings emphasize the functional importance of insulin–melatonin relationship mediated via orchestra between MT1 and MT2 receptor of hepatic and the pancreatic beta cell. Investigation further suggest that pineal gland with its enhanced synthesis of the synchronizing hormone melatonin which in turn may modulate its own receptor MT1 and MT2 which further cross talks with the insulin pathway and hence compensating normal insulin secretion.

Authors contributions

SR was involved in the conception and design of the study. YAH was involved in the biochemical, molecular evaluation and preparation of histological slides. YAH was also involved in data analysis, interpretation and drafting. Authors read and approved the final manuscript.

Declaration of competing interest

The authors declare there is no conflict of interest.

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