Life Sciences 295 (2022) 120382

Contents lists available at ScienceDirect

Life Sciences

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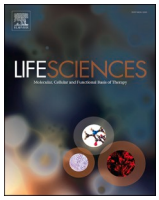

Calorie restriction potentiates the therapeutic potential of GABA in managing type 2 diabetes in a mouse model

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ARTICLE INFO

Keywords:
Type 2 diabetes
Obesity
Calorie restriction
γ-Aminobutyric acid
β-Cell regeneration

ABSTRACT

Dysfunctional adipocytes/β-cells advance type 2 diabetes (T2D). Calorie restriction (CR) improves insulin sensitivity and fasting blood glucose (FBG) levels, while γ-aminobutyric acid (GABA) exerts regenerative effects. The impact of therapies was assessed by a high-fat diet (HFD) + streptozotocin (STZ) induced T2D mouse model. The mice were fed a CR diet (30% reduction of HFD) and treated with GABA (2.5 mg/kg i.p) for 5 weeks. Standard protocols were used to assess metabolic parameters. The mRNA expression was monitored by SYBR Green-qPCR in the targeted tissues. Oxygen consumption rate in the mitochondrial complexes was evaluated by oxytherm clark-type oxygen electrode. Pancreatic β-cell regeneration and apoptosis were analysed by immunohistochemistry. CR + GABA combination therapy showed improved metabolic parameters compared to the monotherapies. We have observed improved transcript levels of *G6Pase*, *PEPCK*, *Glycogen Phosphorylase*, *GLUT2* and *GCK* in liver; *ACC* and *ATGL* in adipose tissue. Also increased *SIRT-1*, *PGC-1α* and *TFAM* expression; up-regulated mitochondrial complexes I-III activities were observed. We have seen increased BrdU/Insulin and PDX1/Ngn3/Insulin co-positive cells in CR + GABA treated group with a reduction in apoptotic marker (TUNEL/Insulin co-positive cells). Our results indicate that CR in combination with GABA ameliorates T2D in HFD + STZ treated mice by GABA induced β-cell regeneration, and CR mediated insulin sensitivity.

1. Introduction

Dysfunctional adipocytes and β-cells are involved in advancing obesity-induced type 2 diabetes (T2D) [1–2]. Several aspects regulate the progression of T2D, such as genetic predisposition [2–11], excess calorie intake [1,12] and sedentary lifestyle [12], all of which cause persistent hyperglycemia and eventual β-cell apoptosis. Even though there have been many recent management therapies for T2D, none can withstand the worsening condition of β-cell loss. This wide gap has triggered the search for alternative remedies that can regenerate β-cells from either stem cells or existing β-cells and bring about insulin sensitivity.

Calorie restriction (CR), an effective dietary intervention for T2D, has been associated with longevity. CR reduces caloric intake, typically by 20–40% of ad libitum consumption, while maintaining adequate intake of protein and micronutrients to avoid malnutrition [13]. CR attenuates the degree of oxidative stress [14,15] and increases the transcript levels of genes involved in mitochondrial function and biogenesis [15]. It also improves insulin sensitivity [13,14], FBG, other cardiometabolic risk factors, and reduces pro-inflammatory adipokines and total cholesterol [16].

γ-aminobutyric acid (GABA) has emerged as a new antidiabetic dietary supplement. GABA, a neurotransmitter, is secreted by the central nervous system (CNS) and pancreatic β-cells. It mediates protective and regenerative effects by reducing β-cell apoptosis and increasing its replication rate [17,18]. GABA exerts its action via two primary receptors $GABA_A$ and $GABA_B$, which regulates various functions in the pancreatic islets [19]. GABA secreted from β-cells binds to $GABA_A$Rs on

Abbreviations: T2D, Type 2 Diabetes; FBG, Fasting Blood Glucose; TC, Total Cholesterol; HDL, High Density Lipoprotein; TG, Triglycerides; LDL, Low Density Lipoprotein; BMI, Body Mass Index; HFD, High Fat Diet; CR, Calorie Restriction; STZ, streptozotocin; GABA, γ-Aminobutyric Acid.

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https://doi.org/10.1016/j.lfs.2022.120382
Received 13 September 2021; Received in revised form 24 January 2022; Accepted 3 February 2022
Available online 7 February 2022

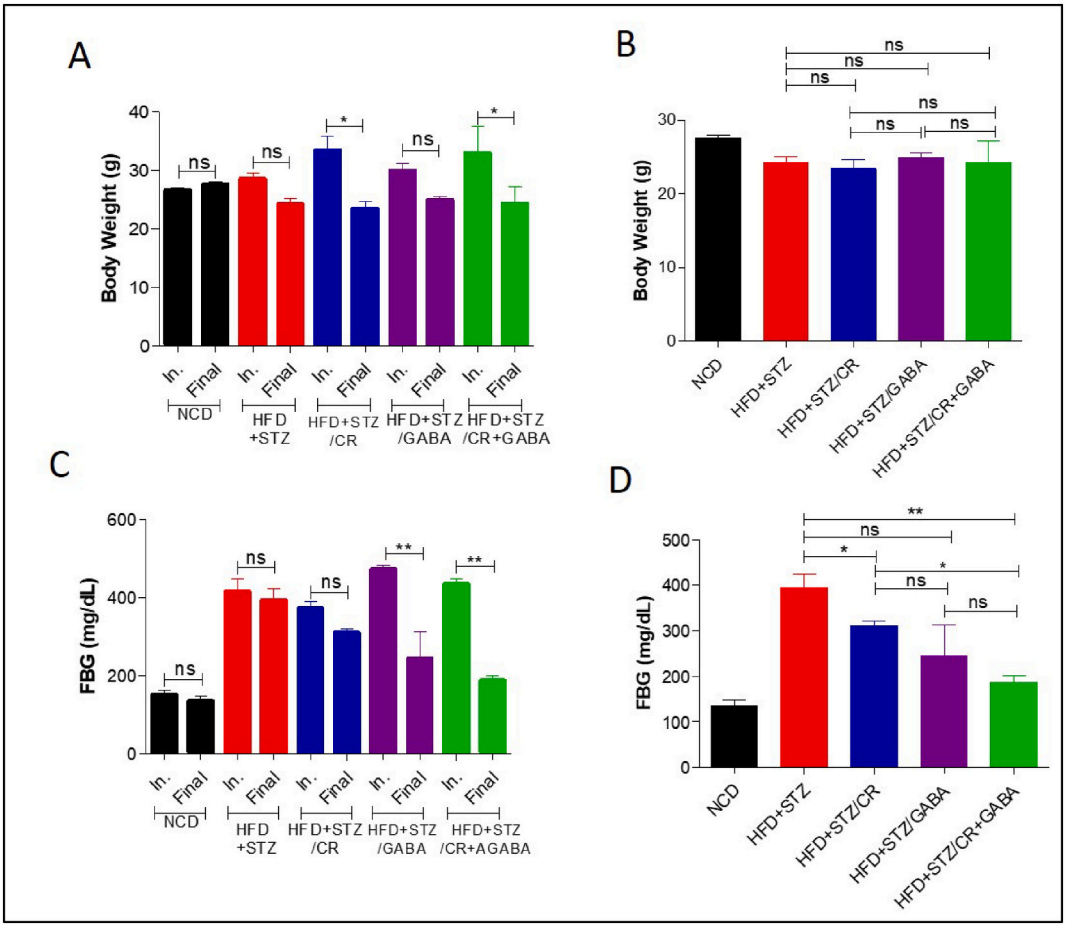

Fig. 1. Body weight and fasting blood glucose levels. A) A significant decrease in BW was observed in the CR and CR + GABA treated groups as compared to HFD + STZ. B) No difference in body weight was observed between all the groups. C) FBG levels in mice treated with GABA and CR + GABA were significantly reduced post-treatment. D) A significant reduction in FBG levels was observed in CR + GABA as compared to CR and HFD + STZ treated groups (ns = non-significant, $*p = 0.05$; $**p < 0.01$, $n = 4$–5/group).

α-cells, leading to an influx of Cl^- and membrane hyperpolarization, thereby inhibiting glucagon release. Unlike mature neurons and α-cells, GABA induces membrane depolarization via $GABA_A$Rs on the β-cells, and enhances insulin secretion in the presence of glucose [19]. GABA has shown type 1 diabetes ameliorating effect by suppressing Treg cells in long-term therapy where GABA has demonstrated induction of β-cell regeneration by proliferation and transdifferentiation [20,21].

We have investigated the combinatorial effect of CR and GABA in the T2D mouse model induced by high-fat diet (HFD) + streptozotocin (HFD + STZ). To evaluate the effect of CR and GABA on multiple targets and regulatory pathways, we assessed i) insulin sensitivity, glucose tolerance, and metabolic profile, ii) transcript levels of key genes involved in hepatic glucoregulation and lipid metabolism in adipose tissue (AT) and mitochondrial biogenesis in skeletal muscle (SK), iii) ETC complexes I, II and III activities in SK, and iv) regenerative markers in β-cells.

2. Materials and methods

2.1. Animals and experimental strategy

2.1.1. Ethics statement

The experimental procedures were approved by the Institutional Ethical Committee for Animal Research (IECHR), Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat, India (FS/IECHR/2016-9).

2.1.2. Animals

Male C57BL/6 mice (~8–10 weeks old, approximately 20–22 g) were procured from ACTREC, Mumbai. They were housed at 23.0 ± 1 °C with a 12-h light/dark cycle and acclimatised for one week. These animals had free access to standard chow/HFD/CR diet (Keval Sales Corporation, Vadodara, India) and water.

2.1.3. Development of T2D mouse model

The mice were divided into two groups. The control group ($n = 6$) was fed with a normal chow diet (NCD). Obesity can induce multiple damaging effects on peripheral tissues. Working on similar lines, mice ($n = 25$) were fed with HFD for 20 weeks to induce obesity and insulin resistance. After 20 weeks, these mice received three consecutive doses of 40 mg/kg BW STZ i.p. (MP Biomedicals, India) to induce β-cell loss. The HFD + STZ model is characterized by a significantly increased weight (≥30 g), hyperglycemia (FBG ≥300 mg/dL), hyperlipidemia, insulin resistance and β-cell loss [22].

2.1.4. Treatment

The HFD + STZ treated animals were divided randomly into four groups (4–5 mice/group): 1. Diabetic control (HFD + STZ) 2. CR diet-fed (30% reduction of HFD) [23,24] 3. GABA treated (2.5 mg/kg bw i.p) (Sigma–Aldrich, United States) [25] and 4. CR + GABA treated (30% reduction of HFD + GABA, 2.5 mg/kg bw i.p). Treatment was given daily for five weeks along with 100 mg/kg bw i.p BrdU (MP Biomedicals, India) on alternate days. CR diet comprised of 39% casein, 21% lard, 19% sucrose, 16% vitamin-mineral premix, and 5% cellulose. The

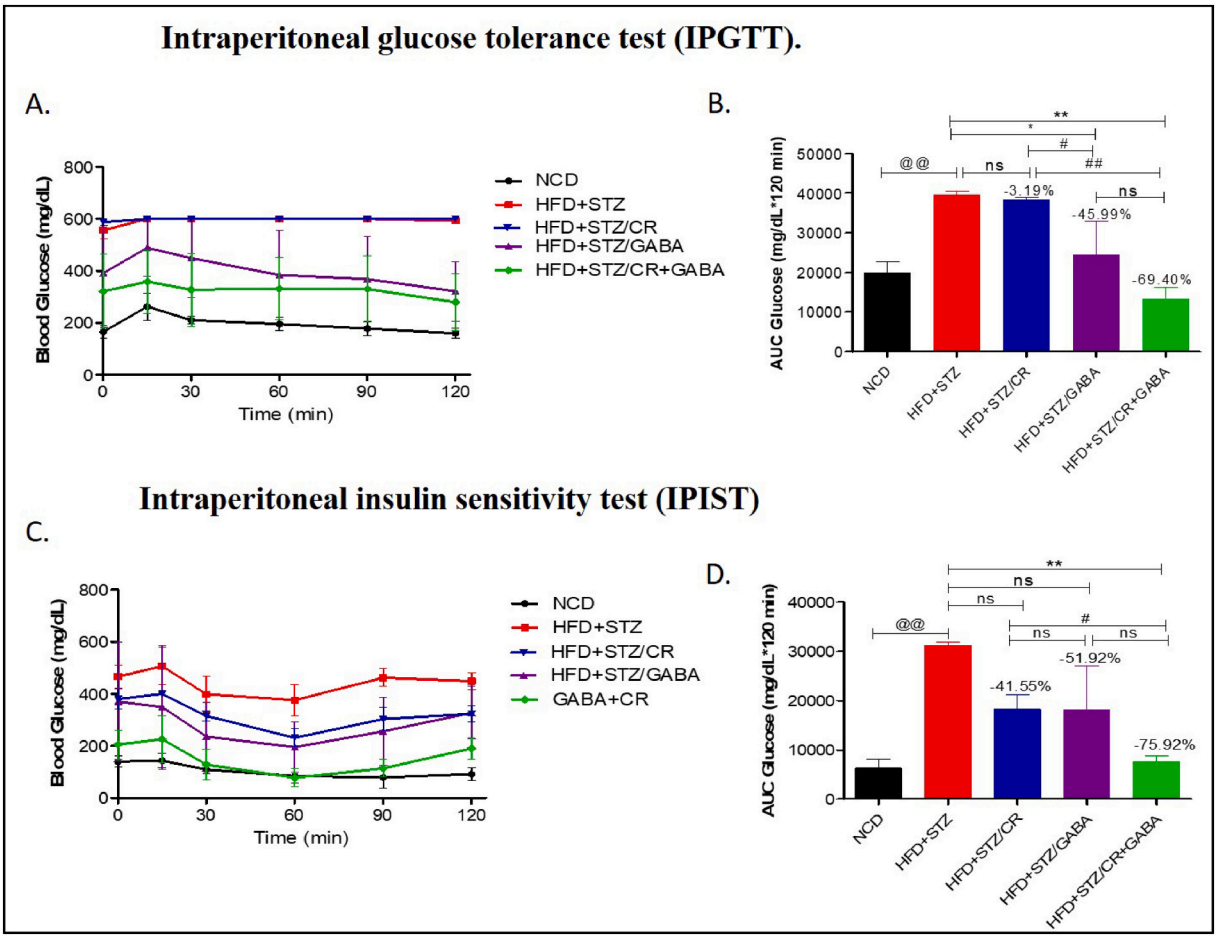

Fig. 2. IGTT and IPIST. A) Blood glucose levels in the GABA and CR + GABA groups were significantly lower than CR and HFD + STZ mice at 60, 90 and 120 mins of glucose administration. B) AUC 0–120 curve indicated improved glucose tolerance in GABA and CR + GABA treated groups compared to HFD + STZ group. C) Blood glucose levels in CR + GABA were significantly lower than in CR, GABA and HFD + STZ groups at 60, 90 and 120 mins of insulin administration. D) AUC 0–120 curve in mice treated with CR + GABA combination was significantly lower than in HFD + STZ group. (ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs HFD + STZ; $^{@}p < 0.05$, $^{@@}p < 0.01$ vs NCD; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs CR n = 4–5/ group).

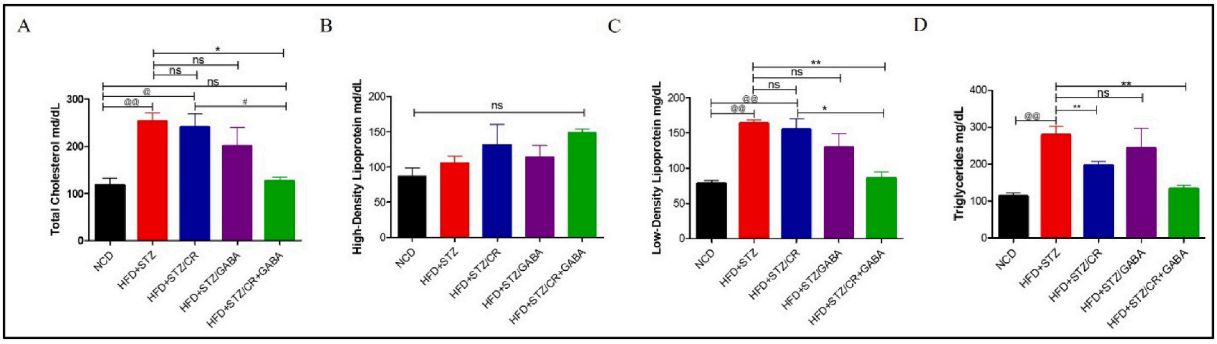

Fig. 3. Plasma lipid levels. A-D) TC, TG, and LDL levels were reduced in CR + GABA group as compared to HFD + STZ group while no difference was observed in the rest of the treatment groups (ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, vs HFD + STZ; $^{@}p < 0.05$, $^{@@}p < 0.01$ vs NCD; $^{\#}p < 0.05$, vs CR n = 4–5/ group).

timeline is shown in fig. S1.

2.2. Metabolic and biochemical assessment

2.2.1. Metabolic profiling

FBG was measured by tail snipping weekly using glucometer (TRUEresult, Nipro, India) along with body weight (BW) as well as food and water intake. At the end of the experiment, 1 ml of blood was collected from the orbital sinus for the biochemical assays after 5 h of fasting. Plasma was separated and used for assessing lipid profile [total cholesterol (TC), triglycerides (TG) and high-density lipoprotein (HDL)] by using commercial kits (Reckon Diagnostics P. Ltd., India). Friedewald's (1972) formula was used for calculating low-density lipoprotein (LDL).

2.2.2. Intraperitoneal glucose tolerance test (IPGTT) and intraperitoneal insulin sensitivity test (IPIST)

IPGTT and IPIST were performed to evaluate glucose tolerance and

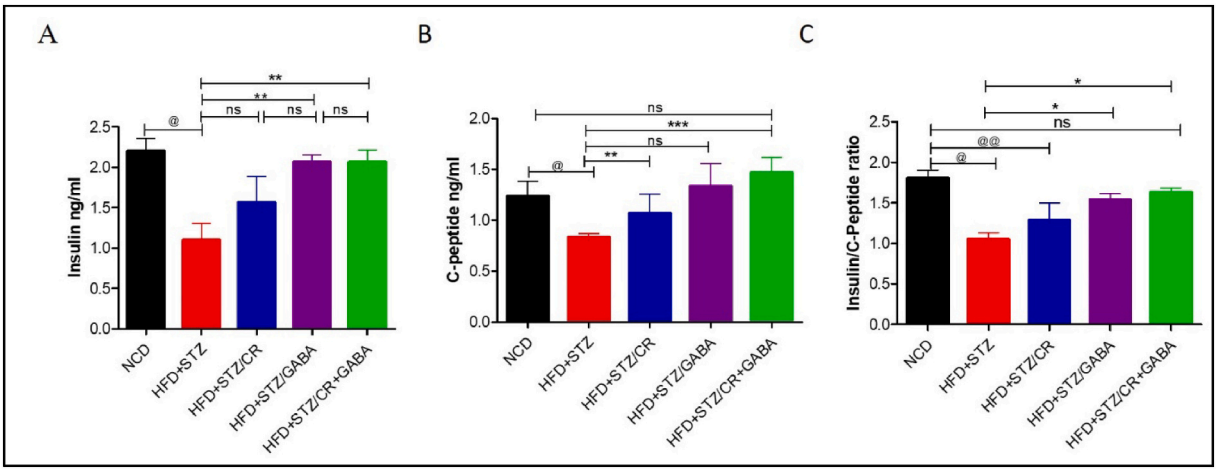

Fig. 4. Plasma insulin, c-peptide levels and insulin/c-peptide ratio. (A & B) Increased insulin and c-peptide levels were observed in GABA & CR + GABA treated groups as compared to HFD + STZ. C) A significant increase in the insulin/c-peptide ratio was observed in GABA & CR + GABA treated groups as compared to HFD + STZ group (ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs HFD + STZ; $^{@}p < 0.05$, $^{@@}p < 0.01$ vs NCD; n = 4–5/ group).

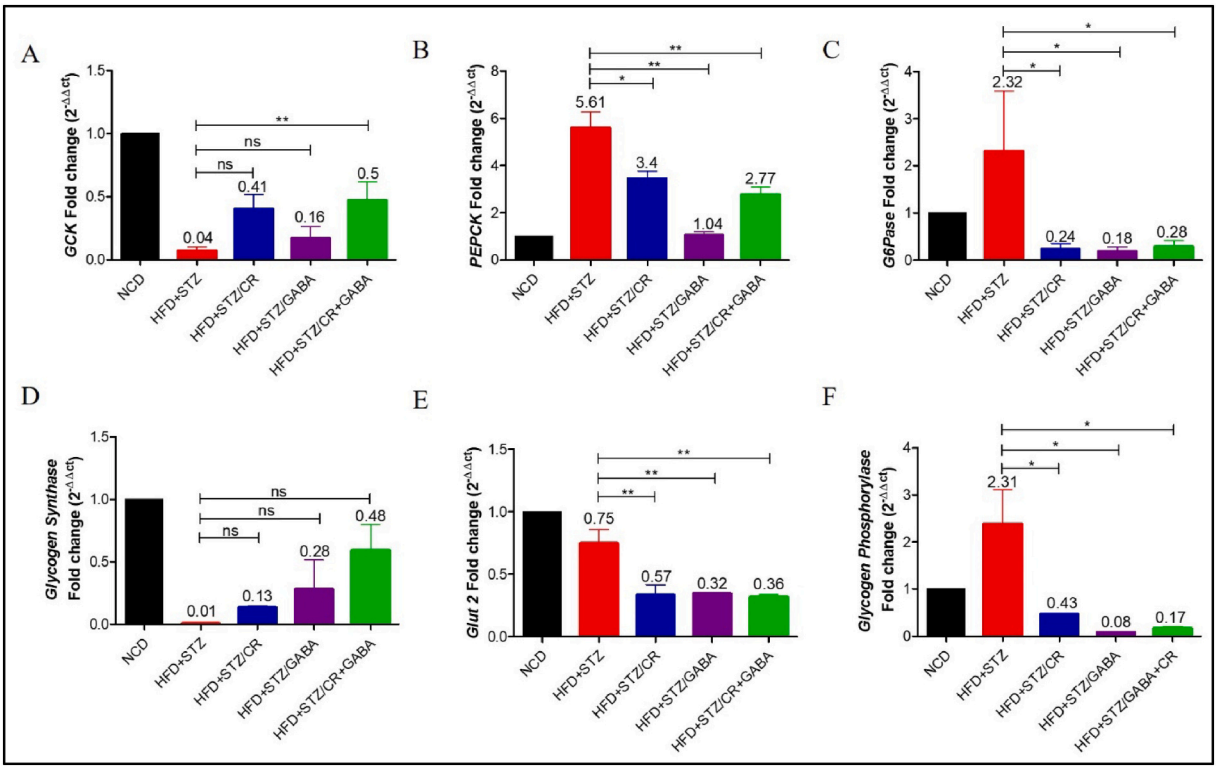

Fig. 5. Transcript levels of glucoregulatory enzymes in the liver: A. *GCK* fold change. After normalisation with GAPDH expression, a 0.5-fold increase in *GCK* transcript levels was observed in the CR + GABA group as compared to the HFD + STZ group. B & C. Fold change in *PEPCK* & *G6Pase*. GABA, CR and CR + GABA showed a significant 1-fold, 3-fold and 2.7 fold decrease in *PEPCK*. GABA, CR, and CR + GABA showed a significant 0.18-fold, 0.24-fold and 0.28-fold decrease in G6Pase expression compared to the HFD + STZ group. D-E.*Glycogen synthase*, *GLUT2* fold change. No significant change was observed in *glycogen synthase,* whereas *GLUT2* expression decreased in all the treated groups compared to the HFD + STZ group. F. Fold change in *Glycogen phosphorylase*. A significant 0.08-fold, 0.43-fold and 0.17-fold reduction in *glycogen phosphorylase* expression was observed in the treatment groups compared to the HFD + STZ group. (ns = non-significant, $^{*}p < 0.05$, $^{**}p < 0.01$; $^{***}p < 0.001$, n = 4–5/group).

insulin sensitivity at the end of 5 weeks of drug treatment. Mice were fasted for 6 h and injected with glucose (2 g/kg BW i.p) or insulin (0.5 U/kg BW i.p) for IPGTT and IPIST [26] respectively. Blood glucose levels were measured at the specified time points 0, 30, 60, 90 and 120 mins after glucose or insulin injection using glucometer.

2.2.3. Assessment of insulin and C-peptide levels

The plasma levels of insulin and C-peptide in the experimental animals were measured using commercially available ELISA kits (RayBio, USA). All the plasma estimations were carried out in duplicate to ensure the coefficient of variation (CV) was below 10%.

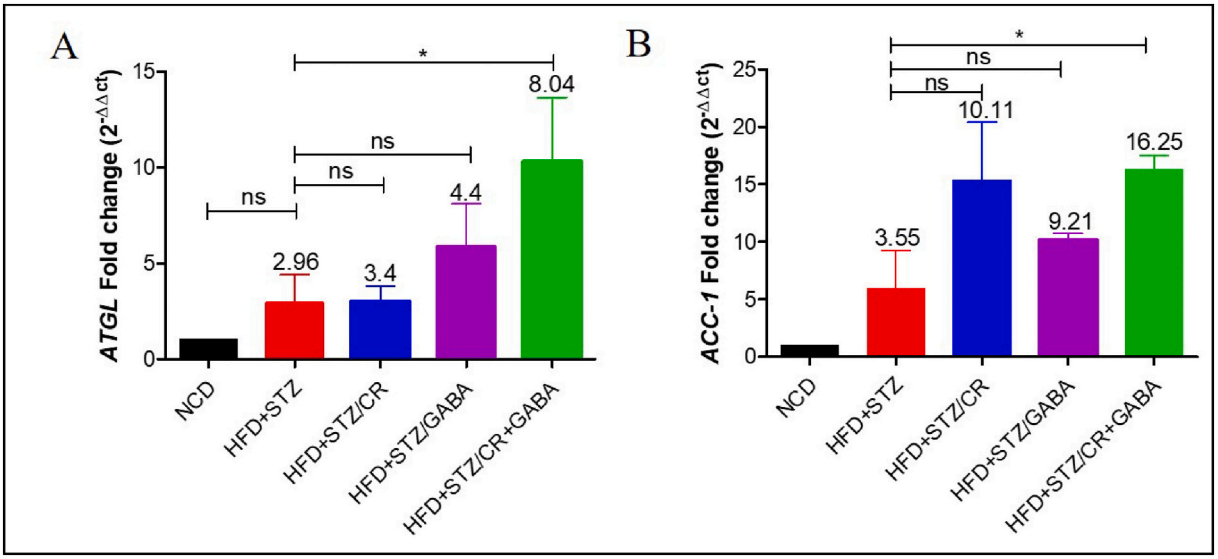

Fig. 6. Transcript levels of lipid metabolism markers in the adipose tissue. A) Fold change in *ATGL* mRNA. A significant 8-fold increase in *ATGL* expression could be seen in the CR + GABA combination group as compared to the HFD + STZ group. B) Fold change in *ACC-1* mRNA. A significant 16-fold increase in *ACC-1* expression was seen in the CR + GABA group compared to the HFD + STZ group. (*$p < 0.05$, ns = non-significant; $p > 0.05$) (n = 4–5/group).

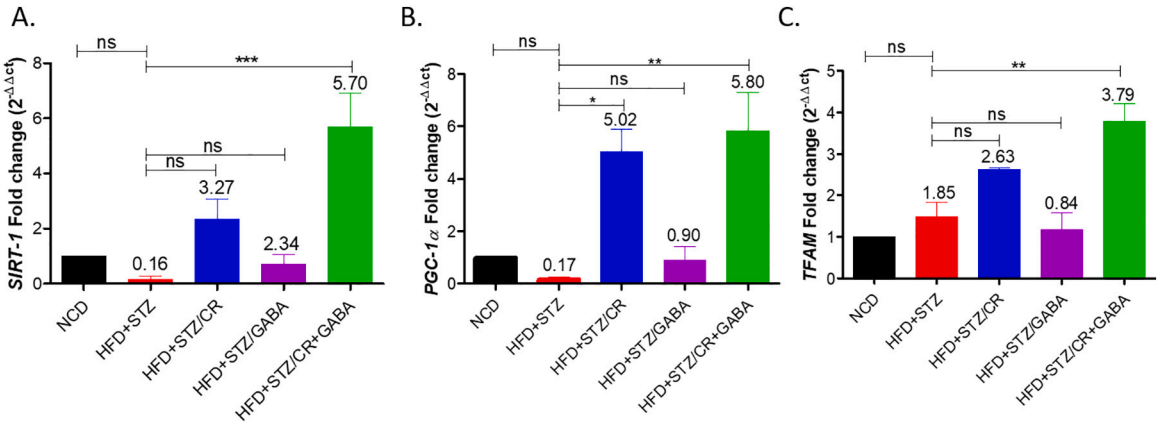

Fig. 7. Transcript levels of mitochondrial biogenesis markers in the skeletal muscle. A) Fold change in *SIRT-1* mRNA. After normalisation with GAPDH expression, 5.7 fold increase in *SIRT-1* expression was observed in the CR + GABA treated group as compared to HFD + STZ group. B) *PGC-1α* mRNA fold change. A significant increase in *PGC-1* expression was observed in CR (4.7-fold) and CR + GABA (5.8-fold) treated groups as compared to HFD + STZ group. C) *TFAM* mRNA fold change. A significant increase in *TFAM* expression (3.7-fold) was observed in the CR + GABA treated group as compared to HFD + STZ group. (*$p < 0.05$, **$p < 0.01$, ns = non-significant; $p > 0.05$) (n = 4–5/group).

2.3. Gene expression profiling

After sacrificing the mice, peripheral tissues such as liver, AT and SK were dissected and stored in RNAlater™ Stabilization Solution (Thermo Fisher Scientific, USA). Total RNA was extracted by the Trizol method as described previously [8]. The expression of targeted genes and *GAPDH* transcripts were monitored by LightCycler®480 Real-time PCR (Roche Diagnostics GmbH, Germany) using gene-specific primers (Eurofins, India) [Table S1]. Expression of *GAPDH* gene was used as a reference. Real-time PCR was performed as described previously [5].

2.4. Estimation of oxygen consumption rate (OCR) of mitochondria

Skeletal muscle was isolated from left thigh of the mouse and mitochondria were isolated from using mitochondria isolation kit (Thermo Scientific TM, Catalog no. 89801) using manufacturer's protocol. The isolated mitochondria were resuspended in mitochondria respiration buffer (80 mM KCl, 0.1% BSA, 50 mM HEPES, 2 mM $MgCl_2$ and 2.5 mM KH_2PO_4; pH 7.2). Outer membrane integrity of the isolated mitochondria was evaluated by impermeability to exogenous cytochrome *c* which was constantly >95%. The activities of respiratory chain complexes I-III were recorded using 100 mM Pyruvate & 800 mM Malate (complex I), 1 M Succinate (complex II) and 10 mM α-glycerophosphate (complex III) and, the protein concentration was estimated by Bradford method. OCR was determined by measuring the amount of oxygen (nmol) consumed, divided by the time elapsed (min) and the amount of protein present in the assay [27].

2.5. Pancreatic tissue preparation, immunohistochemistry (IHC), assessment of β-cell regeneration and apoptosis

At the end of 5 weeks of drug treatment animals were sacrificed, pancreatic tissues were fixed in 10% formalin and were processed for paraffin embedding. 5 μm sections were cut from the paraffin-embedded blocks. To perform IHC, these sections were deparaffinised in 100% xylene and washed serially in ethanol grades (100%, 95%, 80% and

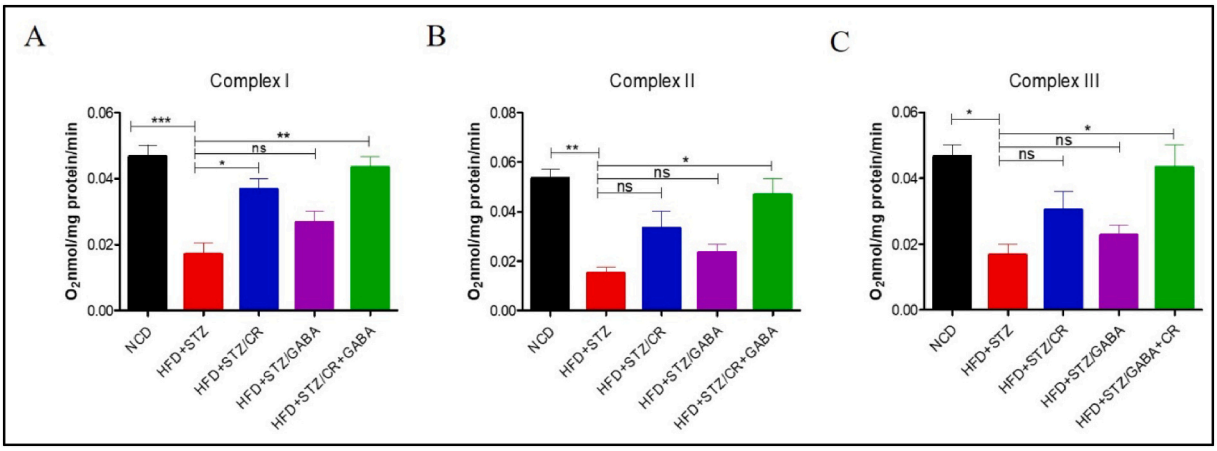

Fig. 8. Estimation of OCR in the skeletal muscle. The activities of complexes I-III were significantly reduced in the HFD + STZ group. A) CR and CR + GABA treated groups showed a significant increase in complex I as compared to HFD + STZ. B—C) CR + GABA treated group showed a significant increase in complexes II and III activities compared to the HFD + STZ group. (ns = non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $n = 4$–5 group).

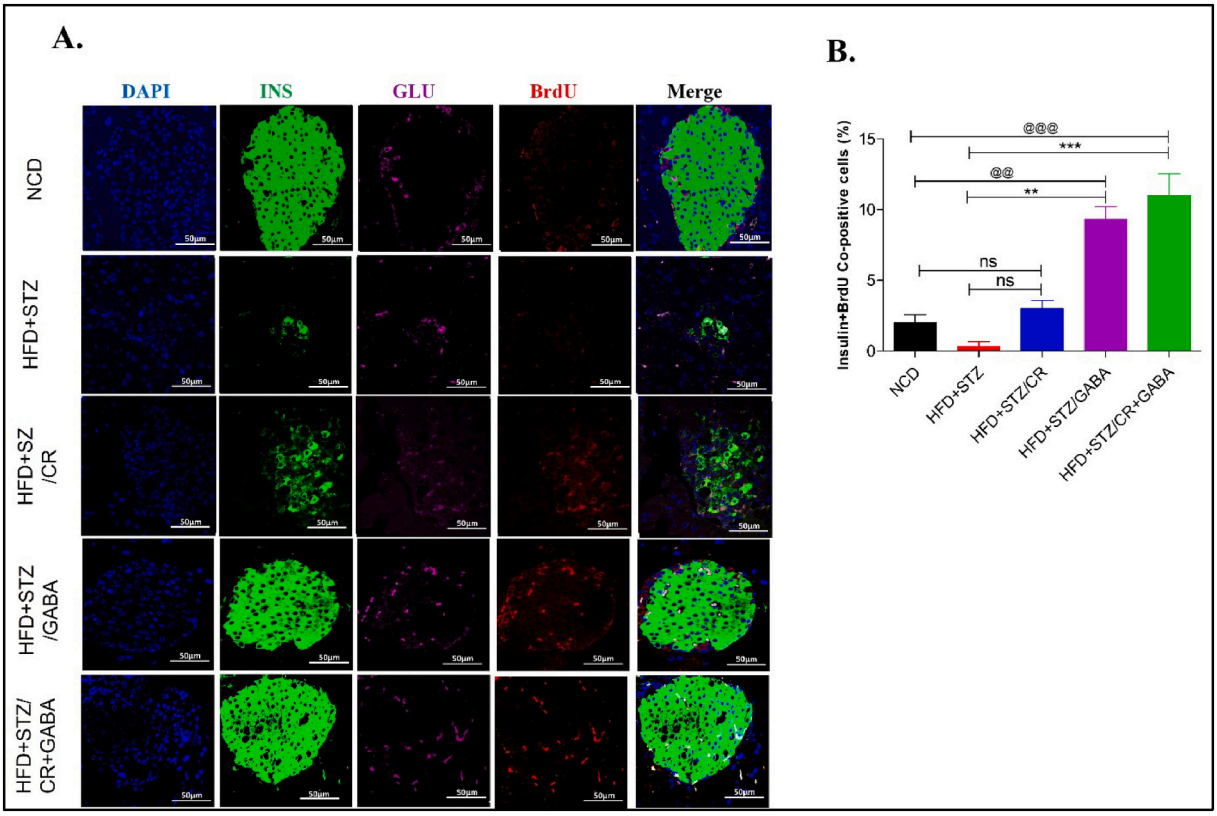

Fig. 9. Assessment of pancreatic β-cell proliferation. (A) Representative immunofluorescence images of pancreatic islets showing insulin (green), glucagon (magenta) and BrdU (red) cells in all the groups. (B) Percentage of Insulin and BrdU co-positive cells. GABA and CR + GABA treated groups showed a significant increase in β-cell proliferation as compared to the HFD + STZ group. However, no significant difference was observed in the CR group. (ns = non-significant, $**p < 0.01$, $***p < 0.001$ vs HFD + STZ; $^{@@}p < 0.01$, $^{@@@}p < 0.001$ vs NCD; $n = 3$/ group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

70%) and proceeded with an antigen retrieval step (1 N HCl at 37 °C for 45 min). Subsequently, these sections were blocked with 5% donkey serum made in phosphate buffer saline (PBS) [Jackson ImmunoResearch Laboratories, Inc. USA], and antibodies were diluted in the blocking reagent. The details of the antibodies are as indicated in table S4. The sections were incubated with primary antibody for 1 h at 37 °C, washed with PBS, and incubated with secondary antibody for 45 min at 37 °C. These sections were stained with anti-fade DAPI (Thermo Fisher Scientific, USA). The sections were covered with a coverslip. The sections were visualised under a confocal microscope (ZEISS LSM, Oberkochen, Germany) at 60×, and the images were processed by Image J (NIH, USA). Immunofluorescence staining (IFS) with anti-insulin and anti-BrdU was used to monitor β-cell proliferation. IFS with anti-insulin, anti-PAX-4 (paired box gene 4), and anti-ARX (Aristaless Related Homeobox) was carried out for α-cell to β-cell transdifferentiation while anti-insulin, anti-NGN-3 (Neurogenin 3) and anti-PDX-1 (pancreas/

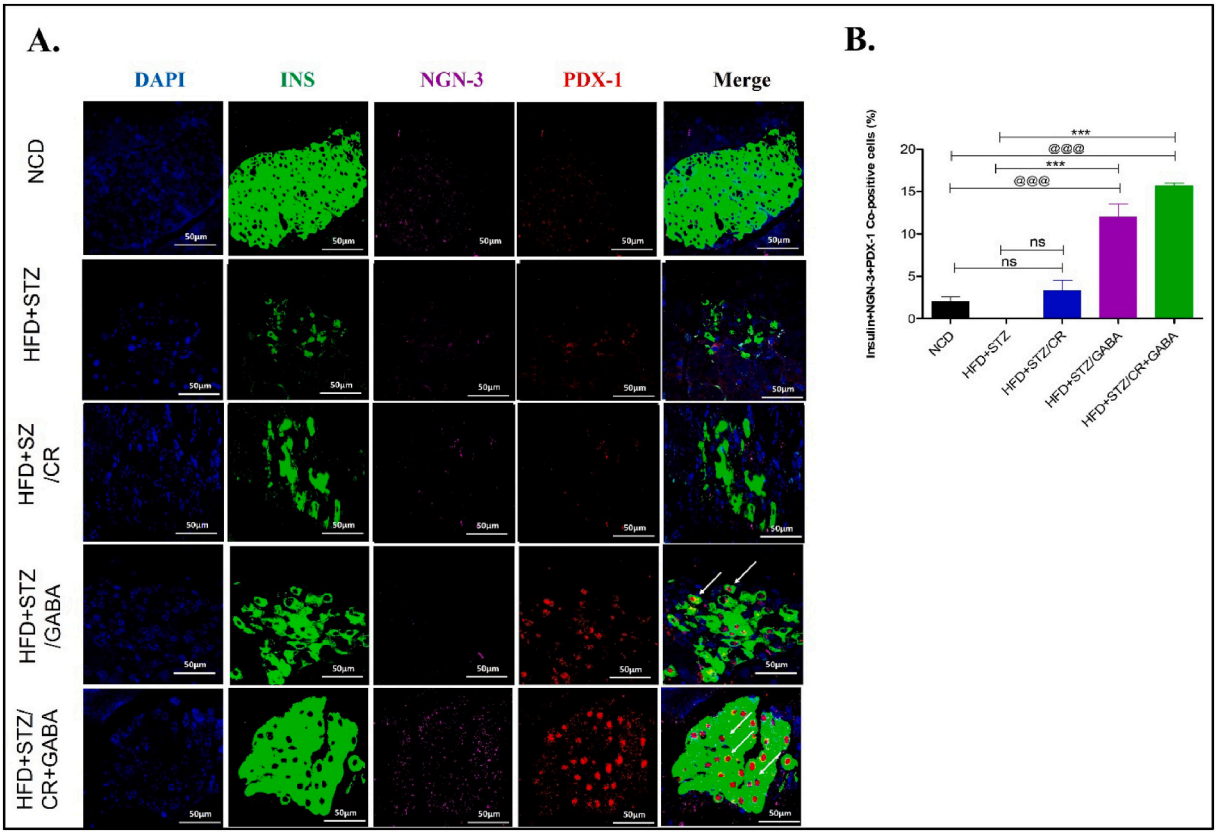

Fig. 10. Assessment of pancreatic β-cell neogenesis. (A) Representative immunofluorescence images of pancreatic islets showing insulin (green), NGN-3 (magenta) and PDX-1 (red) cells in all the groups. (B) Percent of Insulin, NGN-3 and PDX-1 co-positive cells. GABA and CR + GABA treated groups showed a significant increase in β-cell neogenesis as compared to the HFD + STZ group. However, no significant difference was observed in the CR group. (ns = non-significant, $^{***}p < 0.001$ vs HFD + STZ; $^{@@@}p < 0.001$ vs NCD; $n = 3$/ group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

duodenum homeobox protein 1) were considered for β-cell neogenesis. IFS anti-insulin and TUNEL/anti-AIF (Apoptosis Inducing Factor) monitored β-cell death. Results obtained were expressed as the percentage of specified markers for regeneration and death.

2.6. Statistical analyses

The data was analysed and expressed as the mean ± SEM, and $p < 0.05$ was considered statistically significant. The inter-group analysis was carried out by one-way ANOVA, followed by Tukey's test for multiple group analysis. All the analyses were carried out using the Prism software 6 (GraphPad Prism, San Diego, CA, USA).

3. Results

3.1. HFD + STZ induced T2D mouse model establishment

After 20 weeks of HFD treatment, animals turned obese and insulin resistant. There was a significant increase in BW (Table S2a; Fig. S1a&b). After two weeks of STZ administration, the mice turned hyperglycaemic as indicated by FBG levels ≥300 mg/dL (Table S 2b; Figs. S1 c &d).

3.2. Metabolic and biochemical assessment

3.2.1. Metabolic profiling

GABA treated group showed significantly reduced FBG levels ($p < 0.01$), but no change was observed in BW, while CR monotherapy showed improvement in BW ($p < 0.05$) but not in FBG levels. The BW ($p < 0.05$) and FBG ($p < 0.01$) levels of the CR + GABA treated group were significantly reduced at the end of 5 weeks of drug treatment compared to the HFD + STZ group, as shown in Fig. 1 (A and C). We observed non-significant difference between the treated groups for BW (Fig. 1B). However, FBG levels were significantly reduced in CR + GABA treated group as compared to CR monotherapy group (Fig. 1D).

3.2.2. Glucose tolerance and insulin sensitivity

To further test our hypothesis, we performed IPGTT and IPIST post-treatment. CR monotherapy did not show an improvement in glucose tolerance and insulin sensitivity. GABA ($p < 0.05$) and CR + GABA ($p < 0.01$) treated groups showed a significant drop in the blood glucose levels by 120 mins as compared to HFD + STZ group (Fig. 2A). The AUC plot showed a significant increase in glucose tolerance in GABA and CR + GABA treated groups ($p < 0.05$) (Fig. 2B). All the treated groups showed percentage change in glucose levels CR (−3.19%), GABA (−45.99%) and CR + GABA (−69.40%) as compared to HFD + STZ treated group. CR + GABA ($p < 0.01$) treated group showed improved insulin sensitivity as the blood glucose levels were reduced to normoglycemia by 120 mins as compared to HFD + STZ group (Fig. 2C & D). All the treated groups showed percentage change in glucose levels CR (−41.55%), GABA (−51.94%) and CR + GABA (−75.92%) as compared to HFD + STZ treated group.

3.2.3. Lipid profiling

The GABA treated group showed no significant difference in the lipid profile ($p > 0.05$), while CR fed mice ($p < 0.05$) showed reduced TG levels as compared to HFD + STZ group. TC, TG and LDL levels were significantly reduced in CR + GABA treated group ($p < 0.05$) as

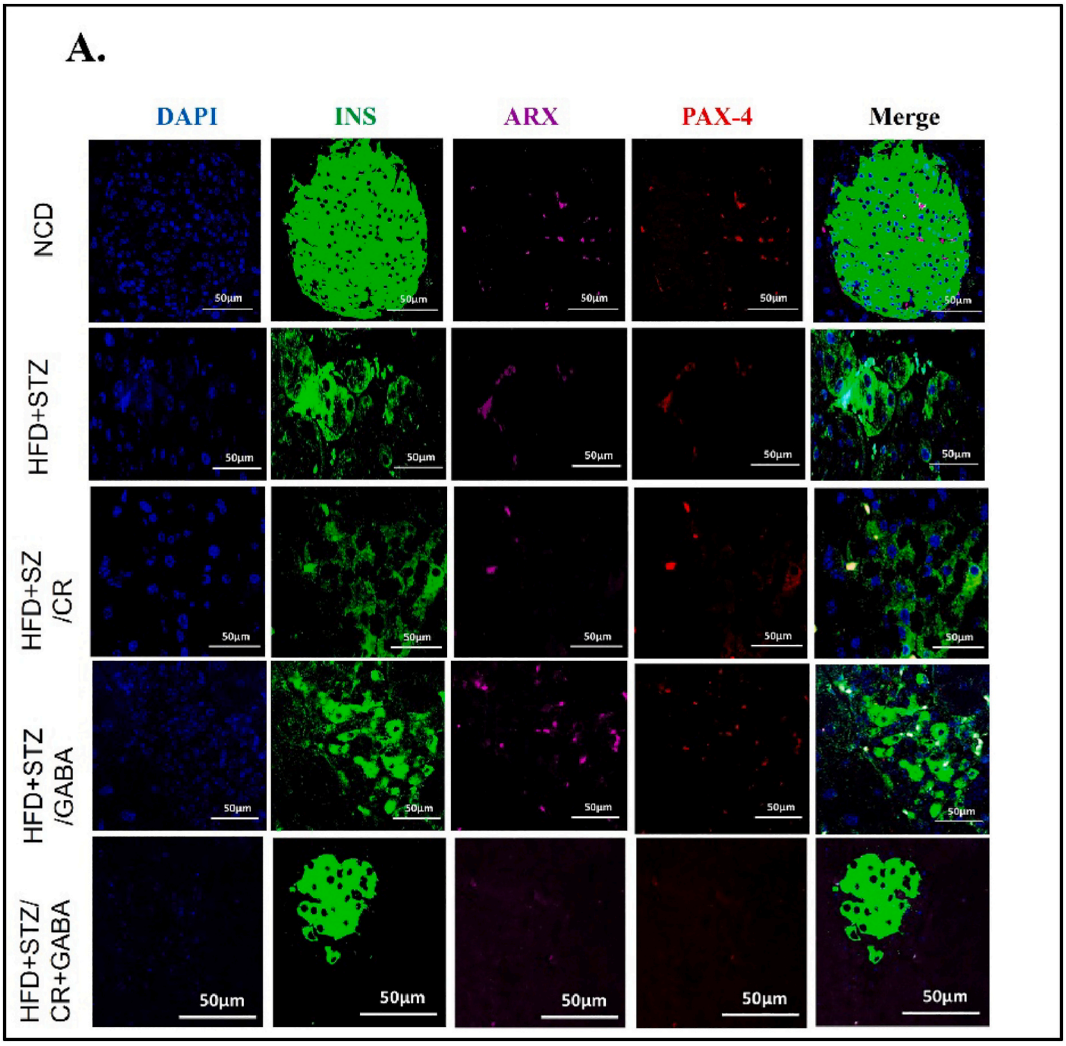

Fig. 11. Assessment of pancreatic β-cell trans-differentiation. (A) Representative immunofluorescence images of pancreatic islets showing insulin (green), ARX-1 (magenta) and PAX-4 (red) cells in all the groups. (ns = non-significant; $n = 3$/ group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

compared to HFD + STZ group (Fig. 3).

3.2.4. Insulin and C-peptide levels

A significant increase in insulin and c-peptide levels along with improved insulin/c-peptide ratio was observed in GABA & CR + GABA ($p < 0.05$) treated groups as compared to HFD + STZ group (Fig. 4 A-C). No significant difference was observed in CR group.

3.3. Gene expression profiling

CR fed mice showed a significant decrease ($p < 0.05$) in the expression of *G6Pase*, *PEPCK* (gluconeogenesis), *GLUT2* and *GP* (glycogenolysis) in the liver (Fig. 5). However, there was no significant difference observed in the expression of *ATGL* and *ACC-1* in adipose tissue of CR fed mice ($p > 0.05$) (Fig. 6). Further, CR fed mice showed a significant increase ($p < 0.05$) in *PGC-1α* transcript levels in skeletal muscle (Fig. 7). GABA treated group showed a significant decrease ($p < 0.05$) in the expression of *phosphoenolpyruvate carboxykinase* (*PEPCK*-gluconeogenesis), glucose-6 phosphatase (*G6Pase*), *glycogen phosphorylase* (*GP*-glycogenolysis) and *glucose transporter-2* (GLUT-2) in the liver (Fig. 5). However, there was no significant difference in the expression of *adipose triglyceride lipase* (*ATGL*-lipolysis) and *acetyl-CoA carboxylase-1* (*ACC-1*-lipogenesis) in the adipose tissue ($p > 0.05$) (Fig. 6). Also, there was no significant difference observed in *sirtuin-1* (*SIRT-1*), *PPARG coactivator 1-α* (*PGC-1α*), and *transcription factor A mitochondrial* (*TFAM*-mitochondrial biogenesis) transcript levels in the skeletal muscle ($p > 0.05$) (Fig. 7). CR + GABA treated group showed a significant decrease ($p < 0.05$) in the expression of *G6Pase*, *PEPCK* (gluconeogenesis), *GLUT2* and *GP* (glycogenolysis) genes, and a significant increase ($p < 0.05$) in *glucokinase* (glycolysis) expression (Fig. 5). Interestingly, lipogenesis got up-regulated as marked by the increased *ACC-1* expression. Consequently, as a compensatory mechanism, lipolysis was also up-regulated, as observed by the increased *ATGL* expression ($p < 0.05$) (Fig. 6). CR + GABA treated group also showed a significant increase ($p < 0.05$) in *SIRT-1*, *PGC-1α* and *TFAM* transcript levels in skeletal muscle (Fig. 7).

3.4. Oxygen consumption rate

The rate of oxygen consumption is a significant indicator of mitochondrial activity. CR fed mice showed a significant increase ($p < 0.05$) in OCR by ETC complex I compared to the HFD + STZ group, indicating improved complex I activity in skeletal muscle. GABA group showed no significant difference in the ETC complex activities in skeletal muscle ($p > 0.05$). The combination therapy group showed significant OCR by ETC complexes I-III as compared to the HFD + STZ group indicating improved complex activities in skeletal muscle ($p < 0.05$) (Fig. 8).

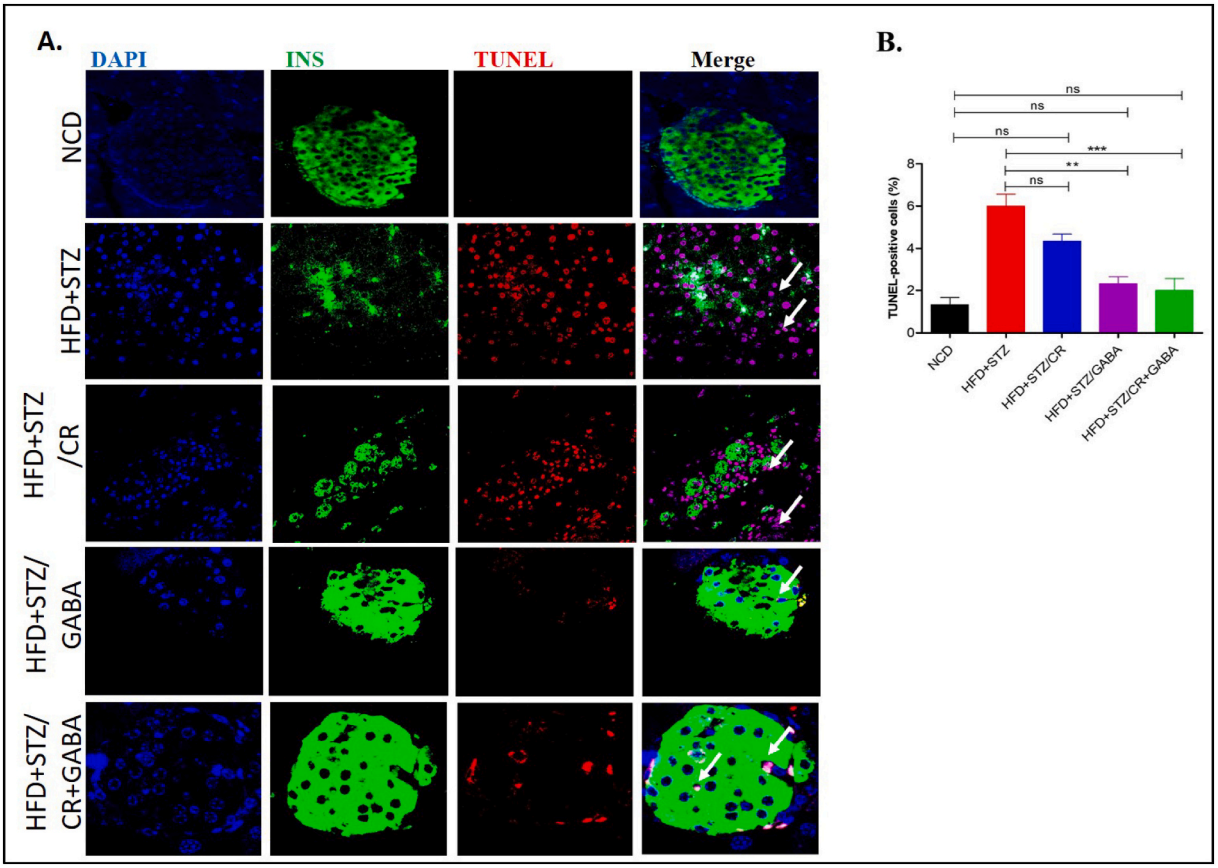

Fig. 12. Assessment of pancreatic β-cell apoptosis: (A) Representative immunofluorescence images of pancreatic islets, showing insulin (green) and TUNEL (red) cells in all the groups. (B) Percent of Insulin and TUNEL co-positive cells. A significant reduction in β-cell apoptosis was observed in GABA and CR + GABA groups compared to the HFD + STZ group. (ns = non-significant, $^{**}p < 0.01$, $^{***}p < 0.001$ vs HFD + STZ; n = 3/group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.5. Pancreatic β-cell regeneration and apoptosis

β-cell proliferation and apoptosis were assessed by IHC microscopy. The GABA treated group showed a significant increase in β-cell proliferation [BrdU/Insulin co-positive cells] (Fig. 9) as compared to NCD ($p < 0.01$) and HFD + STZ treated groups ($p < 0.001$). A similar trend was observed for neogenesis [PDX-1/NGN-3/Insulin co-positive cells] (Fig. 10) as compared to NCD ($p < 0.001$) and HFD + STZ treated groups ($p < 0.001$). Also, GABA treated group showed a significant reduction ($p < 0.01$) in β-cell apoptosis [TUNEL positive cells] (Fig. 12). However, the CR monotherapy group showed no β-cell regeneration, and there was no improvement in β-cell apoptosis ($p > 0.05$) (Fig. 9-12). The CR + GABA treated group showed a significant increase as compared to NCD and HFD + STZ in β-cell proliferation ($p < 0.001$; $p < 0.001$) (Fig. 9) and neogenesis ($p < 0.001$; $p < 0.001$) (Fig. 10). The CR + GABA treated group showed significantly reduced β-cell apoptosis as compared to HFD + STZ ($p < 0.001$) (Fig. 12). The GABA and CR monotherapies, along with the GABA+CR group, did not show transdifferentiation (ARX/PAX4/Insulin co-positive cells) ($p > 0.05$) (Fig. 11). Further, to validate β-cell apoptosis we used AIF as a marker. However, no AIF translocation was observed in any of the groups (Fig. 13).

4. Discussion

The management of T2D involves two main approaches: i) increasing insulin secretion from the β-cells and ii) increasing insulin-mediated glucose uptake by the peripheral tissues. Patients develop tolerance against the existing line of treatment within a few years, which poses a challenge for developing new medicines. These drugs also lead to many side-effects [1]. Thus, combination therapies involving naturally existing biomolecules are the way forward to overcome the complications of T2D. Therefore, the present study was designed to determine the therapeutic potential of a combination of CR and GABA in the T2D mouse model.

CR is a potential dietary intervention emphasizing caloric management in terms of fats, carbohydrates and proteins with sufficient vitamins and minerals. CR promotes insulin sensitivity, mitochondrial biogenesis and functionality [13,15]. Mice fed with 30–40% of the ad libitum calorie deficit diet showed increased life expectancy, robustness against toxicity and stress [28]. Further data on similar lines showed CR's role in reducing body weight and lipid profile markers when the therapy was prolonged [29]. Several reports have explained CR's role as an activator of the SIRT1-PGC-1α signaling pathway at the transcriptional level [30,33]. CR modulates lipid metabolism via the SIRT-1 signaling pathway and reduces intracellular diacylglycerol species [31]. In the liver, PGC-1α may regulate the gluconeogenic pathway; however, the molecular mechanism remains unknown [32].

Interestingly, we have also seen similar observations of action at multiple sites with CR monotherapy reducing body weight and triglyceride levels and improving insulin sensitivity besides reducing gluconeogenesis and glycogenolysis. Civitarese et al. studied the molecular mechanism of CR in mitochondrial functionality. They explained an increased mRNA expression of the genes involved in mitochondrial biogenesis (viz. *SIRT1*, *PGC-1α* and *TFAM*), with SIRT1 being the direct regulator of the pathway [32]. Increased mitochondrial biogenesis and oxygen consumption rate by ETC complex I in our CR monotherapy group find agreement with their findings.

GABA has a proven role in islet-cell hormone homeostasis,

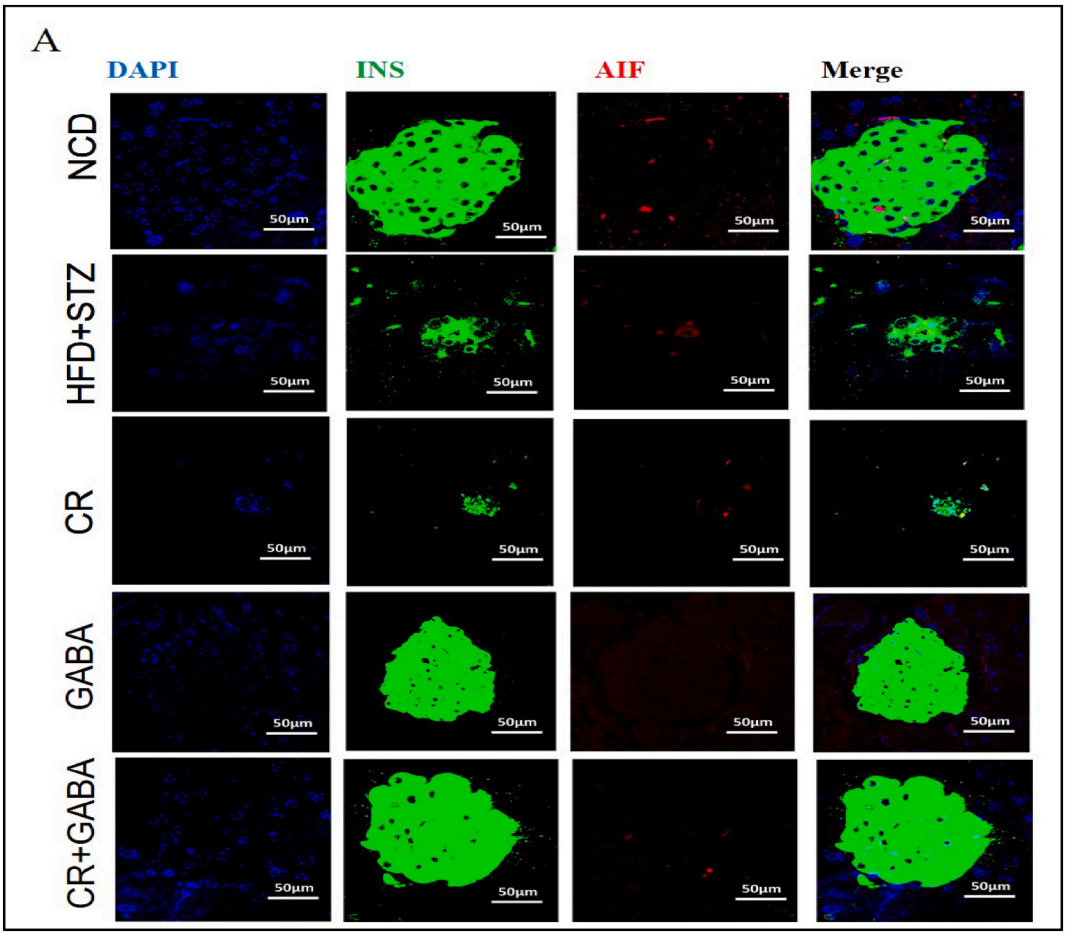

Fig. 13. Assessment of AIF translocation: (A) Representative immunofluorescence images of pancreatic islets, showing insulin (green) and AIF (red) cells in all the groups. No significant translocation of AIF was observed in any of the groups. (n = 3/group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preserving the β-cell mass, suppressing immune reactions and consequent apoptosis [19,34]. The food sources of GABA are potatoes, tomatoes and brown rice, while microorganisms like *E. coli, Lactococcus lactis* produce it [35,36]. In the present study, GABA monotherapy effectively reduces FBG levels, improves glucose tolerance, increases insulin and c-peptide levels and reduces gluconeogenesis and glycogenolysis. GABAnergic system functions in many tissues, including the peripheral tissues. In the liver, $GABA_AR$ regulates PI3/Akt activities that maintain hepatocyte survival and *PGC-1α* expression. Thus, GABA is one of the multiple factors responsible for regulating hepatic glucose metabolism, i.e. gluconeogenic and glycogenolytic pathways [37]. Fibroblast growth factor 21 (FGF-21) predominantly expressed in the liver under normal condition also induces *PGC-1α* expression. It leads to the regulation of lipolysis in the adipose tissue forming liver-adipose tissue crosstalk [38]. The positive effect of GABA treatment stands documented in T1D and T2D murine models. GABA therapy protects NOD animals from diabetes, and a similar effect is reported in various in-vivo models [20,17]. Also, GABA regulates cytokine secretion from human PBMCs and suppresses β-cell-reactive CD8+ CTLs in T1D models [19,17], proposing the role of GABA as an immunosuppressant. GABA reportedly acts as an inducer of α-to-β-like cell conversion in-vivo upon prolonged exposure in the STZ-induced mouse model [19]. Although no β-cell transdifferentiation is seen in our GABA-treated group, a significant increase in β-cell proliferation and neogenesis and reduced β-cell apoptosis were observed. The individual effects of CR and GABA on the multiple pathways and tissues diminished the adverse actions of T2D pathophysiology when given in combination. Our results suggest that the combination treatment improves glucose homeostasis by increasing insulin sensitivity and glucose tolerance. CR enhances insulin sensitivity by improving the mitochondrial function at two levels, elevating the expression of *SIRT1*, *PGC-1α* and *TFAM* and increasing mitochondrial complex I-III activities. GABA therapy boosts insulin levels by inducing β-cell proliferation and neogenesis and contributes to the existing depositary. GABA also enhances the metabolic profile by reducing BW, FBG levels, triglycerides, total cholesterol, and LDL levels. As mentioned earlier, the liver-adipose tissue crosstalk regulates these pathways at the transcriptional level. CR + GABA treated group shows a significant decrease in the expression of *G6Pase* and *PEPCK* (gluconeogenesis), *GLUT2* and *Glycogen Phosphorylase* (glycogenolysis), and an increase in *glucokinase* (glycolysis). Interestingly, lipogenesis seems up-regulated as marked by the increased *ACC* expression, and consequently, lipolysis also gets up-regulated as a compensatory mechanism as observed by the increased *ATGL* expression. CR + GABA treated group showed a significant increase in β-cell proliferation, neogenesis and reduced β-cell apoptosis. We did not observe AIF translocation for β-cell apoptosis, a marker for caspase-independent cell death. Increased oxidative stress induced by STZ and low levels of antioxidant enzymes in the pancreatic cells activate caspase-3 and caspase-9 pathway to mediate β-cell apoptosis [39–41]. Hence, the HFD + STZ model mimics caspase-dependent β-cell apoptosis. However, further validation is required for caspase mediated cell death pathways.

The current study explores the role of CR and GABA therapies in combination to combat T2D manifestations in a holistic approach. It is one of the first studies to explore the unique combination that targets

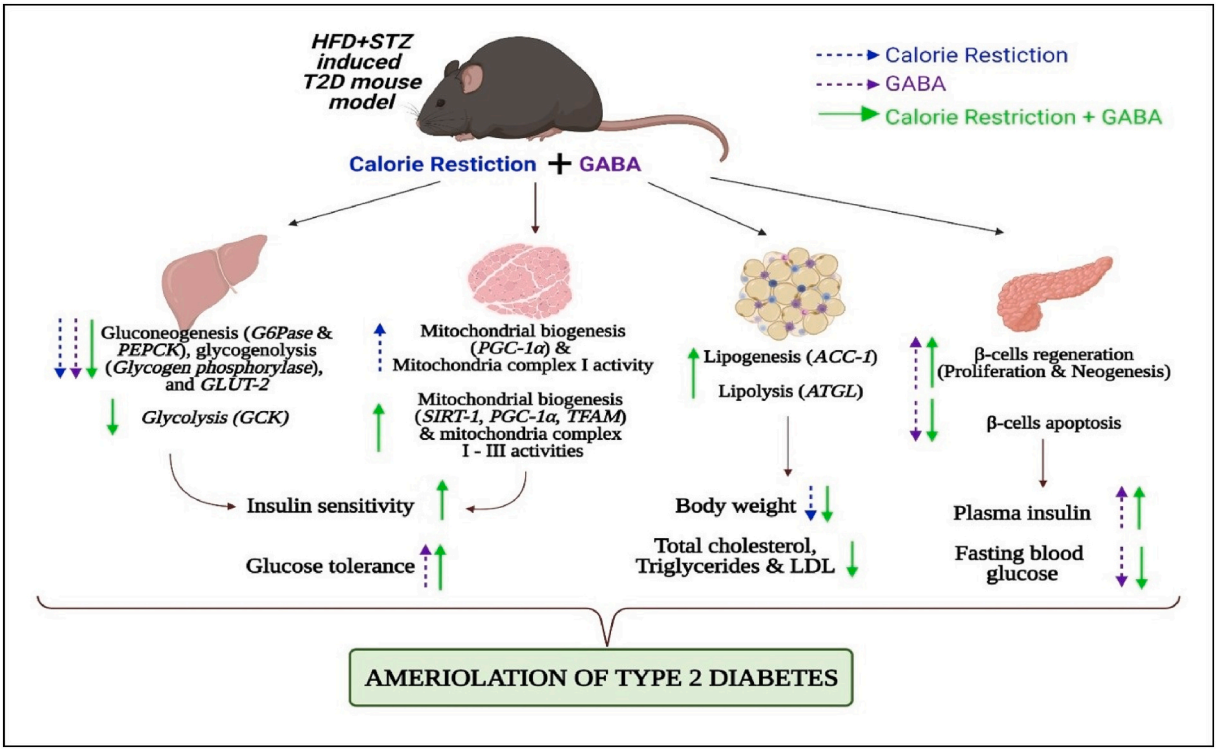

Fig. 14. The effect of GABA, CR and CR + GABA (combination therapy) on amelioration of T2D pathophysiology in HFD + STZ induced T2D mouse model.

multiple tissues and pathways. The T2D mouse model generated for our present study mimics diabetic condition characterized by hyperglycemia, insulin resistance, weight loss, mitochondrial dysfunction and β-cell loss. CR monotherapy targets the parameters of insulin sensitivity, weight loss and mitochondrial functions, while GABA monotherapy focused on β-cell revival pathways. GABA receptors are present on liver and skeletal muscles [42] which might indicate a crosstalk with the effect of CR on these tissues in regulating metabolic pathways. Yet, in-depth studies are required to elucidate the mechanisms. These two therapies synergistically ameliorate the T2D manifestations. However, a few limitations of this study are as follows. First, we used CR as one of our therapies and monitored parameters at the transcriptional level in different peripheral tissues. As CR is a first-line therapy, it is ideal for assessing energy expenditure parameters. Second, our group has not performed cell lineage tracing studies to establish GABA's role in β-cell regeneration. Thus, extensive in vitro and in vivo investigations are needed to address the above burning question and whether the effect was due to the two different signaling cascades, additive stimulus or an undefined underlying mechanism. Thus, the current study suggests that combination therapy with CR and GABA can lead to amelioration of T2D pathophysiology in the T2D mouse model (Fig. 14).

T2D pathophysiology is characterized by insulin resistance and β-cell loss. GABA monotherapy shows reduced FBG levels, improved glucose tolerance, increased insulin and c-peptide levels and decreased gluconeogenesis and glycogenolysis. The GABA treated group also shows a significant increase in β-cell proliferation and neogenesis with significantly reduced β-cell apoptosis. CR diet-fed mice show reduced body weight and triglycerides levels, along with reduced gluconeogenesis and glycogenolysis. These mice also show elevated expression of mitochondrial biogenesis marker and oxygen consumption rate by ETC complexes I compared to the HFD + STZ group. CR diet-fed mice show no β-cell regeneration and no improvement in β-cell apoptosis compared to the HFD + STZ group. GABA+CR treated group shows improved glucose homeostasis by increasing insulin sensitivity and glucose tolerance, enhancing the transcript levels of key markers of glucoregulatory enzymes and lipid metabolism; increasing mitochondrial biogenesis and ETC complex activities. Further, the combination treatment promotes β-cell regeneration and reduces β-cell apoptosis compared to the HFD + STZ group.

5. Conclusions

Our study suggests that CR in combination with GABA therapy could help overcome T2D pathophysiology by their action at multiple sites. CR regulates glucose and lipid metabolism, mitochondrial biogenesis, ETC complex activities, promotes β-cell regeneration and reduces β-cell apoptosis. CR treatment seems part of a first-line therapy that can be used with other drugs like DPP-IV inhibitors or metformin. Moreover, a CR-mimetic, for instance, resveratrol, is available commercially to easily overcome the challenge of strict dietary regimen and protocols. GABA monotherapy works on β-cell regeneration, readily available from natural sources like spinach and tomatoes. An edible vegetable extract can be formulated for diabetic patients without the manifestation of significant side effects. Thus, combination therapy can be useful for effective action at multiple sites and mechanisms to combat T2D pathophysiology. This study needs further investigation at pre-clinical and clinical trial levels to evaluate the commercial viability.

Funding

RB thanks the Department of Biotechnology, New Delhi, India, for the research grant (BT/PR21242/MED/30/1750/2016).

CRediT authorship contribution statement

RB, NR, AVR and RSB conceived the idea. NR designed and performed the experiments; did data acquisition and data analysis; and wrote the original manuscript draft. NP performed a few experiments and data acquisition. SP and RP reviewed and edited the manuscript. RB and AVR contributed to the critical revision and approval of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

We thank Dr. Deepak Sharma, Scientist F, BARC, Mumbai, for providing the confocal facilities. NR thanks University Grants Commission-National Fellowship for higher education for ST students, New Delhi, India, for awarding SRF. RP thanks Council for Scientific and Industrial Research, New Delhi, India, for awarding SRF. Images are created with BioRender.com

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120382.

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Journal of Endocrinological Investigation
https://doi.org/10.1007/s40618-023-02014-6

ORIGINAL ARTICLE

A novel combination of sitagliptin and melatonin ameliorates T2D manifestations: studies on experimental diabetic models

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Received: 5 September 2022 / Accepted: 16 January 2023

Abstract

Introduction Type 2 diabetes (T2D) is an endocrine disorder characterized by hyperglycemia, insulin resistance, dysregulated glucose and lipid metabolism, reduced pancreatic β-cell function and mass, and a reduced incretin effect. Circadian rhythm disruption is associated with increased T2D risk. We have investigated the therapeutic potential of a combination of melatonin (M) and sitagliptin (S), a dipeptidyl peptidase IV (DPP-IV) inhibitor, in the amelioration of T2D manifestations in high-fat diet (HFD) induced T2D mouse model and also on β-cell proliferation under gluco-lipotoxicity stress in vitro.

Methods For in vivo study, mice were fed with HFD for 25 weeks to induce T2D and were treated with monotherapies and S + M for four weeks. For the in vitro study, primary mouse islets were exposed to normal glucose and high glucose + palmitate to induce gluco-lipotoxic stress.

Results Our results suggest that monotherapies and S + M improve metabolic parameters and glyco-lipid metabolism in the liver and adipose tissue, respectively, and improve mitochondrial function in the skeletal muscle. Moreover, it increases peripheral insulin sensitivity. Our in vitro and in vivo studies suggest that β-cell mass was preserved in all the drug-treated groups.

Conclusion The combination treatment is superior to monotherapies in the management of T2D.

Keywords Insulin resistance · Gluco-lipotoxicity · Islets · Glycemic control · Mitochondria · Glyco-lipid metabolism

Abbreviations

T2D	Type 2 diabetes
FBG	Fasting blood glucose
BW	Body weight
TC	Total cholesterol
HDL	High density lipoprotein
TG	Triglycerides
LDL	Low density lipoprotein
BMI	Body mass index
HFD	High fat diet
M	Melatonin
S	Sitagliptin

Introduction

Lifestyle changes, principally, calorie-rich diet intake and nocturnality contribute to an increasing number of metabolic disorders, including obesity and Type 2 Diabetes (T2D) [1]. Disturbances in circadian rhythm are associated with metabolic dysregulation and glucose intolerance in humans [2]. T2D is characterized by insulin resistance, hyperinsulinemia, hyperleptinemia, oxidative stress, inflammation, and metabolic and mitochondrial dysfunction. Impaired insulin action leads to hyperglycemia and dyslipidemia, which causes gluco-lipotoxicity to various cells, including pancreatic β-cells, further contributing to a reduction in β-cell mass and function [3]. The prevalence of T2D is rapidly increasing worldwide. Researchers are coming up with many novel therapeutic approaches including incretin-based therapies that could suppress diabetic manifestations in different organs besides increasing β-cell mass.

Melatonin, a hormone of darkness, is known to reduce obesity and T2D [4–6] by regulating glucose metabolism [7] and lipid metabolism [8, 9] in both rodents and humans. When produced locally in several tissues, including the

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Published online: 24 January 2023

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Article

Repurposing Pitavastatin and L-Glutamine: Replenishing β-Cells in Hyperlipidemic Type 2 Diabetes Mouse Model

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Abstract: Type 2 diabetes (T2D) is associated with obesity and declining β-cells. L-glutamine has been implicated in the amelioration of T2D by virtue of its incretin secretagogue property while, there are mixed reports on pitavastatin's adiponectin potentiating ability. We aimed to investigate the effect of pitavastatin (P), L-glutamine (LG), and combination (P + LG) on glycemic control and β-cell regeneration in a high-fat diet (HFD) + streptozotocin (STZ)-induced T2D mouse model. C57BL6/J mice treated with HFD + STZ were divided into four groups: diabetes control (HFD + STZ), P, LG, and P + LG, while the control group (NCD) was fed with the normal-chow diet. Significant amelioration was observed in the combination therapy as compared to monotherapies in respect of (i) insulin resistance, glucose intolerance, lipid profile, adiponectin levels, and mitochondrial complexes I, II, and III activities, (ii) reduced phosphoenolpyruvate carboxykinase, glucose 6-phophatase, glycogen phosphorylase, and GLUT2 transcript levels with increased glycogen content in the liver, (iii) restoration of insulin receptor 1β, pAkt/Akt, and AdipoR1 protein levels in skeletal muscle, and (iv) significant increase in islet number due to β-cell regeneration and reduced β-cell death. L-glutamine and pitavastatin in combination can ameliorate T2D by inducing β-cell regeneration and regulating glucose homeostasis.

Keywords: obesity; β-cell regeneration; mitochondrial biogenesis; adiponectin; GLP-1

Citation: Palit, S.P.; Patel, R.; Parmar, N.; Rathwa, N.; Dalvi, N.; Ramachandran, A.V.; Begum, R. Repurposing Pitavastatin and L-Glutamine: Replenishing β-Cells in Hyperlipidemic Type 2 Diabetes Mouse Model. *Life* **2023**, *13*, 929. https://doi.org/10.3390/life13040929

Academic Editors: Giuseppe Paolisso and Rachele Garella

Received: 22 December 2022
Revised: 3 February 2023
Accepted: 28 March 2023
Published: 1 April 2023

1. Introduction

Insulin resistance, reduced insulin secretion, dyslipidemia, and β-cell degeneration lay the foundation of type 2 diabetes (T2D). Against a backdrop of obesity or genetic factors the risk for these hallmark foundations increases many folds. While there is little that can be done to reduce the genetic predisposition, having a healthy and active lifestyle can definitely reduce the associated risks. T2D takes several years to set in with the pre-diabetic stage typically being long; however, this stage being asymptomatic, most cases are detected only after diabetes is established [1]. In the prediabetes stage, as insulin resistance sets in and the first phase of insulin response starts to diminish, resulting in β-cells overworking to compensate for the reduced insulin levels. In addition, the excessive circulating lipids in obese conditions create a stressful microenvironment resulting in eventual loss of β-cell mass. Studies have shown that by the time T2D is diagnosed, islet function is already reduced by 50% and β-cell mass by 60% due to the increased stress and accelerated apoptosis [2,3]. Further, in cadaver pancreatic autopsy of obese patients with prediabetes and T2D, Butler et al. demonstrated a 40% reduction in β-cell mass, as compared to obese individuals without T2D [4]. Impaired action or a 50% drop in incretin hormones, glucose-dependent insulinotropic polypeptide (GIP), and GLP-1 have also been reported to result in the decline of β-cell function in type 2 diabetes. This potentiates close to 70% of the meal-induced insulin response in healthy individuals. In this context

BBA - Molecular Cell Research 1869 (2022) 119263

Contents lists available at ScienceDirect

BBA - Molecular Cell Research

journal homepage: www.elsevier.com/locate/bbamcr

Research Paper

A novel therapeutic combination of sitagliptin and melatonin regenerates pancreatic β-cells in mouse and human islets

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ARTICLE INFO

Keywords:
Diabetes
Melatonin
Sitagliptin
β-Cell regeneration
Glucose tolerance

ABSTRACT

Autoimmune-led challenge resulting in β-cell loss is responsible for the development of type 1 diabetes (T1D). Melatonin, a pineal hormone or sitagliptin, a dipeptidyl peptidase IV (DPP-IV) inhibitor, has increased β-cell mass in various diabetic models and has immunoregulatory property. Both β-cell regenerative capacity and melatonin secretion decrease with ageing. Thus, we aimed to investigate the therapeutic potential of melatonin combined with sitagliptin on β-cell regeneration under glucotoxic stress, in the streptozotocin-induced young and old diabetic mouse models, and euglycemic humanized islet transplant mouse model. Our results suggest that combination therapy of sitagliptin and melatonin show an additive effect in inducing mouse β-cell regeneration under glucotoxic stress, and in the human islet transplant mouse model. Further, in the young diabetic mouse model, the monotherapies induce β-cell transdifferentiation and reduce β-cell apoptosis whereas, in the old diabetic mouse model, melatonin and sitagliptin induce β-cell proliferation and β-cell transdifferentiation, and it also reduces β-cell apoptosis. Further, in both the models, combination therapy reduces fasting blood glucose levels, increases plasma insulin levels and glucose tolerance and promotes β-cell proliferation, β-cell transdifferentiation, and reduces β-cell apoptosis. It can be concluded that combination therapy is superior to monotherapies in ameliorating diabetic manifestations, and it can be used as a future therapy for β-cell regeneration in diabetes patients.

1. Introduction

Type 1 diabetes (T1D) is characterized by hyperglycemia due to pancreatic β-cell apoptosis and lack of β-cell regeneration. The most promising treatment for T1D is islet transplantation. However, its application remains restricted due to the lack of donors and lifelong dependence on immunosuppressive drugs [1–3]. Thus, it is crucial to explore novel therapies that would aid in β-cell regeneration either by proliferation, neogenesis, or transdifferentiation for T1D management. Monotherapies often fail in the long run due to other diabetes-related complications and the patient's lifestyle. Hence, combination therapy is recommended for long-term glycemic control.

Incretin-based drugs are currently categorized as novel therapies for type 2 diabetes (T2D) management. These include mainly two categories, GLP-1 receptor agonists (GLP-1RA) and dipeptidyl peptidase-IV (DPP-IV) inhibitors [4]. Both drugs have a low risk of hypoglycemia and function differently to regulate glucose homeostasis. Sitagliptin, a DPP-IV inhibitor, prevents the degradation of incretins (GLP-1 and GIP), elevate their levels, and thereby stimulates insulin secretion. Studies on the T2D rodent model have shown that both classes of drugs promote β-cell proliferation. However, studies demonstrated a nominal beneficial effect of GLP-1RAs in the T1D model, possibly due to its limited immunomodulatory effects [4]. DPP-IV inhibitors with immunomodulatory and glucoregulatory effects are considered as second-line therapy for T2D management, but recently are being studied in T1D models to assess β-cell function and mass. Sitagliptin has been shown to prolong islet graft retention in streptozotocin (STZ)-induced T1D mice by increasing the endogenous levels of incretin hormones [5], and in NOD-mice partially by T-cell modulation [6].

Melatonin, a pineal hormone known as the “hormone of darkness”

Abbreviations: T1D, Type 1 Diabetes; FBG, Fasting Blood Glucose; BW, Body Weight; STZ, Streptozotocin; M, Melatonin; S, Sitagliptin; PDX1, Pancreatic and Duodenal Homeobox 1; ARX1, Aristaless-related homeobox-encoding gene; PAX4, Paired Box 4; NGN3, Neurogenin 3; AIF, Apoptosis Inducing Factor.

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https://doi.org/10.1016/j.bbamcr.2022.119263
Received 17 August 2021; Received in revised form 23 March 2022; Accepted 24 March 2022
Available online 29 March 2022

Biochimie xxx (xxxx) xxx

Contents lists available at ScienceDirect

Biochimie

journal homepage: www.elsevier.com/locate/biochi

Diabetes mellitus and melatonin: Where are we?

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ARTICLE INFO

Article history:
Received 19 July 2021
Received in revised form
7 December 2021
Accepted 4 January 2022
Available online xxx

Keywords:
Melatonin
Diabetes therapeutics
Diabetes complications
Cellular stress
β-cell regeneration

ABSTRACT

Diabetes mellitus (DM) and diabetes-related complications are amongst the leading causes of mortality worldwide. The international diabetes federation (IDF) has estimated 592 million people to suffer from DM by 2035. Hence, finding a novel biomolecule that can effectively aid diabetes management is vital, as other existing drugs have numerous side effects.

Melatonin, a pineal hormone having antioxidative and anti-inflammatory properties, has been implicated in circadian dysrhythmia-linked DM. Reduced levels of melatonin and a functional link between melatonin and insulin are implicated in the pathogenesis of type 2 diabetes (T2D). Additionally, genomic studies revealed that rare variants in melatonin receptor 1b (*MTNR1B*) are also associated with impaired glucose tolerance and increased risk of T2D. Moreover, exogenous melatonin treatment in cell lines, rodent models, and diabetic patients has shown a potent effect in alleviating diabetes and other related complications. This highlights the role of melatonin in glucose homeostasis. However, there are also contradictory reports on the effects of melatonin supplementation. Thus, it is essential to explore if melatonin can be taken from bench to bedside for diabetes management.

This review summarizes the therapeutic potential of melatonin in various diabetic models and whether it can be considered a safe drug for managing diabetic complications and diabetic manifestations like oxidative stress, inflammation, ER stress, mitochondrial dysfunction, metabolic dysregulation, etc.

Contents

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https://doi.org/10.1016/j.biochi.2022.01.001
0300-9084/

Please cite this article as: R. Patel, N. Parmar, S. Pramanik Palit *et al.*, Diabetes mellitus and melatonin: Where are we?, Biochimie, https://doi.org/10.1016/j.biochi.2022.01.001

Nutrition, Metabolism & Cardiovascular Diseases (2020) **30**, 1870–1881

Available online at www.sciencedirect.com

Nutrition, Metabolism & Cardiovascular Diseases

journal homepage: www.elsevier.com/locate/nmcd

REVIEW

β-cell replenishment: Possible curative approaches for diabetes mellitus

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Received 4 April 2020; received in revised form 2 August 2020; accepted 3 August 2020
Handling Editor: S. Piro
Available online 11 August 2020

KEYWORDS
Pancreas;
β-cell apoptosis;
β-cell regeneration;
Islet neogenesis;
α-to-β trans-differentiation;
Regenerative medicine

Abstract *Aims:* Diabetes mellitus (DM) is a disorder of heterogeneous etiology marked by persistent hyperglycemia. Exogenous insulin is the only treatment for type 1 diabetes (T1D). Islet transplantation is a potential long cure for T1D but is disapproved due to the possibility of immune rejection in the later stage. The approaches used for treating type 2 diabetes (T2D) include diet restrictions, weight management and pharmacological interventions. These procedures have not been able to boost the quality of life for diabetic patients owing to the complexity of the disorder.
Data synthesis: Hence, research has embarked on permanent ways of managing, or even curing the disease. One of the possible approaches to restore the pancreas with new glucose-responsive β-cells is by their regeneration. Regeneration of β-cells include islet neogenesis, dedifferentiation, and trans-differentiation of the already differentiated cells.
Conclusions: This review briefly describes the islet development, functions of β-cells, mechanism and factors involved in β-cell death. It further elaborates on the potential of the existing and possible therapeutic modalities involved in the *in-vivo* replenishment of β-cells with a focus on exercise, diet, hormones, small molecules, and phytochemicals.

Introduction

Diabetes mellitus

Diabetes mellitus (DM) is a metabolic disorder characterized by persistent hyperglycemia and, the number of individuals with diabetes has continued to grow over the years. It is mainly classified into type 1 diabetes (T1D) and type 2 diabetes (T2D). Other rare forms of diabetes are directly inherited [1]. T1D constitutes less than 10% of the total cases of diabetes worldwide and is triggered by

Abbreviations: T1D, type 1 diabetes; T2D, type 2 diabetes; FBG, fasting blood glucose; PDX-1, pancreatic and duodenal homeobox 1; NKX6.1, NK6 homeobox 1; NKX2.2, NK2 homeobox 2; GLUT2, glucose transporter 2; ChREBP, carbohydrate response element-binding protein; CR, calorie restriction; GLP-1, glucagon-like peptide-1; GIP, gastric inhibitory polypeptide; PPAR-γ, peroxisome proliferator-activated receptor-γ; GABA, γ-AminoButyric Acid; TZD, thiazolidinediones; DYRK, dual-specificity tyrosine phosphorylation-regulated kinase; DPP IV, dipeptidyl peptidase-IV; Tregs, regulatory T cells.

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https://doi.org/10.1016/j.numecd.2020.08.006

Life Sciences 243 (2020) 117285

Contents lists available at ScienceDirect

Life Sciences

journal homepage: www.elsevier.com/locate/lifescie

Intron specific polymorphic site of *vaspin* gene along with vaspin circulatory levels can influence pathophysiology of type 2 diabetes

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ARTICLE INFO

Keywords:
Obesity
Dyslipidemia
Single nucleotide polymorphism
Genotype-phenotype correlation
Haplotype
Adipokine

ABSTRACT

Vaspin, an insulin-sensitizing adipokine, has been associated with type 2 diabetes (T2D). The present study aimed to investigate the distribution of genotypes and high-risk alleles of *vaspin* genetic variants (rs77060950 G/T and rs2236242 A/T), in Gujarat subpopulation (India). Genomic DNA isolated from PBMCs was used to genotype *vaspin* polymorphisms by PCR-RFLP and ARMS-PCR from 502 controls and 478 patients. RNA isolated from visceral adipose tissue (VAT) of 22 controls and 20 patients was used to assess *vaspin* transcript levels by qPCR while the vaspin titre of the subjects was assayed using ELISA. Phenotypic characteristics of Fasting Blood Glucose (FBG), BMI and plasma lipid profile were estimated and analyzed for the genotype-phenotype correlation. We identified a significant association of rs2236242 A/T with T2D as the TT genotype conferred a 3.087-fold increased risk. The TT genotype showed association with increased FBG, BMI and Triglycerides levels. Increased GA, GT and TA haplotype frequencies, decreased VAT transcript and vaspin protein levels in T2D patients was observed, which were further negatively correlated with FBG and BMI. In conclusion, rs2274907 A/T polymorphism is strongly associated with reduced *vaspin* transcript and protein levels, and related metabolic alterations that may play a role in the advancement of T2D.

1. Introduction

Central obesity, an integral part of the metabolic syndrome, has long been viewed as a risk factor for type 2 diabetes mellitus (T2D). Adipocytes produce many biomolecules, collectively known as adipokines, playing a key role in metabolism, inflammation, and immunity. Since the discovery of leptin, many other adipokines have been discovered forming the crux of homeostasis between the anti- and pro-inflammatory macrophages [1]. Vaspin, a member of serpin A12, was initially discovered in visceral adipose tissue (VAT) of Otsuka Long-Evans Tokushima fatty rat [2,3]. It is an anti-inflammatory adipokine reported to inhibit kallikrein 7 (a protease degrading insulin). It promotes cell proliferation, inhibits apoptosis and ameliorates ER stress in-vitro [3,4]. There are also a few studies establishing the favourable effect of exogenous recombinant vaspin on insulin sensitivity and glucose tolerance [2,5].

In humans, reduced vaspin protein levels have been correlated with increased Body Mass Index (BMI) and reduced insulin sensitivity in adults [6,7], and obese women having Polycystic Ovary Syndrome (PCOS) [8,9]. Thus, the emerging line of evidence supports the concept of vaspin playing a significant role in the progression towards obesity induced T2D. *Vaspin* consists of 6 exons and 5 introns and is located on chromosome 14q32.13. Single nucleotide polymorphisms (SNPs) of *vaspin* are well explored of which, intronic polymorphic sites (intron 2 rs77060950 G/T and intron 4 rs2236242 A/T) have been investigated in relation to various diseases like T2D [10,11], PCOS [12], Metabolic Syndrome [13,14], Coronary Artery Disease (CAD) [15], Nonalcoholic Fatty Liver Disease (NAFLD) [16], obesity [17], and End Stage Renal Disease (ESRD) [18].

The predictions for India indicate that cases of T2D will rise to 74.9 million by 2030 [19] with the Gujarat population being the second highest [20]. We have reported the genetic predisposition of *TNF-α*, *resistin* and *omentin-1* in T2D [21–23]. We, thus, aimed to investigate the distribution of genotypes and high-risk alleles of *vaspin* present in

Abbreviations: T2D, type 2 diabetes; FBG, Fasting Blood Glucose; BMI, Body Mass Index; TC, Total Cholesterol; HDL, High-Density Lipoprotein; TG, Triglycerides; LDL, Low-Density Lipoprotein; PCR-RFLP, Polymerase Chain Reaction-Restriction Fragment Length Polymorphism; ARMS-PCR, Amplification Refractory Mutation System-Polymerase Chain Reaction

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https://doi.org/10.1016/j.lfs.2020.117285
Received 22 October 2019; Received in revised form 29 December 2019; Accepted 5 January 2020
Available online 08 January 2020
0024-3205/