Nano-curcumin safely prevents streptozotocin-induced inflammation and apoptosis in pancreatic ß-cells for effective management of type 1 diabetes mellitus

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Short title: nCUR to manage type 1 diabetes mellitus

Abstract

Background and Purpose: Approaches to prevent selective and progressive loss of insulin-producing ß-cells in Type 1 diabetes mellitus (T1DM) will help conquer this prevalent and devastating disease. Curcumin (CUR), a natural anti-inflammatory, suppresses diabetes associated inflammation and cell death. However, very high doses have been tested owing to poor oral bioavailability, making it difficult to translate to the clinic.

Experimental approach: We recently prepared biodegradable nanosystems encapsulating curcumin (nCUR), resulting in at least 9-fold improvement in oral bioavailability. In the current study, we tested nCUR's ability to prevent streptozotocin (STZ)-induced inflammation and apoptosis in pancreatic islet/ß-cells.

Key Results: Nonfasted rats pretreated with 10 or 50 mg/kg nCUR 6 hours prior to STZ challenge had up to 37% reduction in the glucose levels, while plain CUR (50 mg/kg) results in 12% reduction. This is owing to nCUR's ability to prevent islet/β-cell death evident from TUNEL assay, and H&E staining. Both CUR and nCUR significantly decreased levels of inflammatory cytokines in pancreatic tissue homogenates that correlated well with minimal histiocytic infiltration. The nCUR, rather than CUR pre-treatment prevented 8-oxo-2'-deoxyguanosine (8-oxo-dG), a sensitive biomarker of reactive oxygen species (ROS)-induced DNA damage in pancreas. Our data in normal rodents indicates that 28 days daily dosing with nCUR (25 to 100 mg/kg) did not cause any deleterious health issues by the carrier.

Conclusions & Implications: Together, these data indicate a potentially translatable dose of nCUR that is safe and efficacious in improving the ß-cell function, possibly preventing T1DM.

Abbreviations: Allograft inflammatory factor 1 (AIF-1); Anti-interleukin-1β (anti-IL-1β); Bright field microscope (BFM); Curcumin (CUR); Dynamic light scattering system (DLS); Granulocyte-colony-stimulating factor (G-CSF); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Human Equivalent Doses (HED); Interferon gamma (IFNγ) Interleukin-1 beta (IL-1β); Ionized calcium binding adaptor molecule 1 (Iba1); Nano-curcumin (nCUR); 8-Oxo-2'-deoxyguanosine (8-oxo-dG); Pharmacodynamic (PD); Pharmacokinetic (PK); Polyvinyl alcohol (PVA); Rat pancreatic β-insulinoma cells (RIN-m5F); Reactive oxygen species (ROS); Streptozotocin (STZ); Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL); Tumor necrosis factor-alpha (TNFα); Type 1 diabetes mellitus (T1DM).

Table of Links

LIGANDS	
Natural products or derivatives	**Cytokines and growth factors**
[Curcumin](#)	TNF-alpha
	IL-6
	IL-1ß

Introduction

Type 1 diabetes mellitus (T1DM) is a heterogeneous disorder characterized by primary or secondary islet damage, making the beta (β) cells antigenic, prompting T-lymphocyte infiltration and production of pro-inflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNFα), and interferon gamma (IFNγ) (Atkinson, Eisenbarth & Michels, 2014; Eisenbarth, 1986; Hamilton, 2008; Meah et al., 2016; Skyler, 2013). The clinical manifestations of T1DM are only evident when nearly 80% of the β cell mass has already been destroyed. To date there is no record of success with preventative strategies (Brooks-Worrell & Palmer, 2013;

Reimann et al., 2009; Skyler, 2015; Skyler & Pugliese, 2013; Wherrett, 2014). Over the years, we have witnessed a plethora of developments in T1DM therapies that include insulin replacement, immunosuppression, antigen-specific and cell-based approaches. T1DM prevention in high risk individuals remains the highest priority where the goal is to maintain endogenous β-cell function (Creusot, Battaglia, Roncarolo & Fathman, 2016; Li, Cheng & Zhou, 2016). Therefore, protection of β-cells from cell death is considered as a new therapeutic target, (Ardestani & Maedler, 2016; Imai, Dobrian, Morris, Taylor-Fishwick & Nadler, 2016; Roy, Janal, Crosby & Donnelly, 2016; Srimal & Dhawan, 1973) where natural and safe anti-inflammatories, such as curcumin (CUR), (Castro et al., 2014; Srimal & Dhawan, 1973) can perform better than some of the biologics (e.g., Canakinumab, a fully human anti-interleukin-1β (anti-IL-1β) monoclonal antibody [IgG-1κ class]) tested in trials with limited success (Cabrera et al., 2016). A critical barrier in the clinical translation of CUR is its limited oral bioavailability (Shaikh, Ankola, Beniwal, Singh & Kumar, 2009). We have recently reported several studies on CUR encapsulated nanosystem (nCUR) large scale preparation, and their therapeutic evaluation in models of diabetes complications (Grama et al., 2013; Grama, Venkatpurwar, Lamprou & Kumar, 2013; Ratnam, Wadsworth & Kumar, 2011). We herein report the ability of nCUR to prevent streptozotocin (STZ)-induced inflammation and apoptosis in pancreatic islet/β-cells in rats, reflecting in a reduction of glucose levels and oxidative stress.

Materials and Methods

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA) and all other chemicals were obtained from Fisher Scientific (USA) unless otherwise

mentioned. Curcumin (CUR) was purchased from Acros organics (New Jersey, USA). Antibodies were purchased from Thermo Fisher Scientific (Rockford, IL, USA), Santa Cruz (Dallas, Texas), Life technologies, USA and Cell Signaling, USA. DAPI was purchased from Vector Laboratories, USA, TUNEL was purchased from Roche Diagnostics (Mannhem, Germany). Rat insulin ELISA kits were purchased from Mercodia [10-1250-01] (Uppsala, Sweden), ProcartaPlex Rat Th complete panel was purchased from Affymetrix, eBiosciences (14plex EPX140-30120-901, USA) and, Histomount was purchased from Ted Pella Inc. RIN-m5f cells were purchased from ATCC and maintained in RPMI-1640 medium.

Preparation and characterization of nCUR

The nCUR were prepared as previously reported by our laboratory (Grama et al., 2013; Grama, Venkatpurwar, Lamprou & Kumar, 2013; Ratnam, Wadsworth & Kumar, 2011; Shaikh, Ankola, Beniwal, Singh & Kumar, 2009). In brief, polylactide-co-glycolide (PLGA) (500 mg) was dissolved in 20 mL of ethyl acetate, to which 75 mg CUR pre dissolved in 5 mL of ethyl acetate was added then stirred for 1 h at 1,000 rpm on a magnetic stirrer. This organic phase was added dropwise to 50 mL of 1% (w/v) polyvinyl alcohol (PVA) in water forming an oil-in-water emulsion followed by homogenization at 16,000 rpm for 30 min. The emulsion was added to excess water (250 mL) and stirred overnight to evaporate organic solvent. The suspension was centrifuged at 15,000g for 30 min at 4°C. The pellet was re-suspended in water (~15 mL). Sucrose (1.5 g) was dissolved separately in 5 mL of water then added to the re-suspended pellet with the entire suspension volume made to 30 mL. Two milliliters of the suspension was added per 5 mL vial and freeze dried. Three vials from the lot were used to measure particle

size and CUR content using particle size analyzer [Brookhaven dynamic light scattering system (DLS)], and a previously developed HPLC method, respectively (Grama, Venkatpurwar, Lamprou & Kumar, 2013; Shaikh, Ankola, Beniwal, Singh & Kumar, 2009).

In vitro testing of nCUR in rat pancreatic β-insulinoma cells (RIN-m5F)

The RIN-m5f cells were cultured according to the cell bank protocol. The cells ($5x10^4$/well in 24-well plates) were pretreated with various concentrations of CUR or nCUR (10 & 20 μM) for 1 h before challenging with 5 mM STZ (dissolved in citrate buffer, pH 4.5; containing trisodium citrate 0.1 M and citric acid 0.1 M) for 24h. The cells were washed twice with 1% BSA in PBS, 0.5 μg/mL propidium iodide (PI) was added before observation under an EVOS microscope. In another set of experiments, cells were fixed in 4% formaldehyde at room temperature for 30 min followed by washing with PBS. The effects of CUR/nCUR were evaluated by immunocytochemistry by staining fixed cells for caspase-8.

Animal study

All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Texas A&M University, College Station (study-1, efficacy), or, of the University of Strathclyde, Glasgow under the Animals (Scientific Procedures) Act 1986 (UK) (study-2, safety). Three month old male Sprague Dawley (SD) rats (250 ± 15 g, n=6) were used in study-1; two month old male SD rats (200 ± 15 g, n=4) were used in study-2. All animals were fed normal diet *ad libitum* throughout the studies. All end-point adjudication was blinded to dosing.

Experimental protocol

Study 1: After acclimatization for two weeks, the rats (male SD) were pre-treated with a single dose of CUR or nCUR (10 or 50 mg/kg) for 6 h prior to STZ challenge. All rats received a single intraperitoneal injection of 60 mg/kg STZ dissolved in 0.1 M sodium citrate buffer, pH 4.5 after 8h fasting. The glucose levels in nonfasted rats were monitored at 24, 48, 72 h after STZ injection using precision Xtra glucometer (Abbott Diabetes Care Inc, Alameda, CA) and all rats were sacrificed at 72 h post STZ. The control groups received neither CUR nor STZ. Blood and pancreas were collected and processed for analysis of various markers, histological evaluation and immunohistochemistry. The study had the following six groups: Group 1 (Control); Group 2 (STZ) diabetic control (STZ 60mg/kg intraperitoneal injection); Group 3 (STZ + CUR10) 10mg/kg curcumin; Group 4 (STZ + CUR50) 50mg/kg curcumin; Group 5 (STZ + nCUR10) 10mg/kg nano-curcumin; Group 6 (STZ + nCUR50) 50mg/kg nano-curcumin.

Study 2: After acclimatization for two weeks, the rats were treated with CUR 500 mg/kg or nCUR 25, 50, or 100 mg/kg daily by oral gavage for 28 days (Supplement Table S1). The control groups received equal volume of vehicle (water in this case), while nblank group received blank nanoparticles equivalent to the highest dose of CUR (100 mg/kg) for 28 days. Upon termination of the experiment at the end of 28 days, blood and other organs were collected and processed for hematological and histological analyses (detailed procedures and data are presented in Supplementary files).

Blood collection and pancreas separation

Rats in each group on Study-1 were sacrificed as per IACUC protocol at 72h; blood was collected via cardiac puncture in heparinized tubes and centrifuged at 1,620 g for 30 min. The plasma was immediately stored at −80 °C for further use. The pancreas was surgically excised from the rats. These tissues were either stored at −80 °C until further analysis or fixed in 10% buffered formalin for histological assessment.

Preparation of tissue homogenate

A 10% pancreatic tissue homogenate was prepared using a probe sonicator (qsonica) at 30% amplitude for 30 secs, in ice cold homogenizing buffer (phosphate buffer/pH 7.4 supplemented with protease and phosphatase inhibitors). The homogenates were spun down for 30 min at 20,817 g at 4 °C. The supernatant was collected and stored at −80 °C until further use.

Determination of plasma insulin

Plasma insulin levels were determined by using rat insulin ELISA kits.

Multiplex immunoassay for cytokines

The cytokine panel of 14 markers was measured in plasma and pancreas homogenates using a Bio-Plex® MAGPIX™ Multiplex Reader multiplex immunoassay. The markers analyzed included IL-1 alpha, G-CSF, IL-10, IL-17A, IL-1 beta, IL-6, TNF-alpha, IL-4, GM-CSF, IFN-gamma, IL-2, IL-5, IL-13, IL-12p70.

Histological studies

Pancreas from normal and experimental rats were fixed in 10% neutral buffered formalin (NBF) and processed for routine histology and paraffin embedding. Four

micrometer tissue sections were stained with hematoxylin and eosin and evaluated histologically to determine the degree of islet destruction.

Immunohistochemistry to evaluate histiocytic infiltration in pancreatic islets

Sections were incubated on a hot plate for 45-60 min at 60°C, followed by deparaffinizing in xylene for 5 min, and rehydration in decreasing grades of ethanol (100%, 95%, and 70%). Antigen retrieval was performed by boiling the sections using microwave, in 0.01M sodium-citrate buffer pH 6.0 for 10 mins and blocked with blocking solution (3% goat or horse serum in PBS). Slides were washed 3 times with PBS and incubated in PBS containing goat serum with (2µg/mL) anti-Iba1 antibody specific to human, mouse and rat microglia and macrophages at 4°C overnight. Slides were washed 3 times with PBS and the binding of primary antibodies visualized by the corresponding anti-Ig HRP detection kit according to the manufacturers' instructions. Slides were counterstained with Mayer's-hematoxylin (Sigma–Aldrich) and coversliped.

Immunofluorescence of insulin, TUNEL, 8-oxo-dG

Pancreases from normal and experimental rats were fixed in 10% NBF (24h), incubated in increased concentrations of sucrose solution (30%), in and stored in OCT medium at -80°C, until being processed for cryosectioning. Four micrometer cryosections were used for the immunohistochemical staining for insulin, and 8-oxo-dG.

After antigen retrieval, cryosections were immunoreacted with respective primary antibodies (Abs) overnight at 4°C. All steps were preceded by rinsing with PBS (pH 7.4). Immunostaining was performed according to the standard protocol routinely used for immunohistopathology. Primary anti-insulin Ab [clone INS05 (2D11-H5)] was diluted to

1:200. Primary monoclonal Ab to 8-hydroxyguanosine (8OHG), a marker of oxidative damage to nucleic acids was diluted to 1:200. Goat polyclonal anti-rabbit IgG conjugated with Alexa Fluor® 488 (green), 594 (red) were used as the secondary antibody at a dilution of 1:1000. All samples were mounted with Vectashield mounting medium containing DAPI. Goat polyclonal anti-mouse conjugated with Alexa Fluor® 594 IgG was used as the secondary antibody for insulin detection at a dilution of 1:1000 in PBS. TUNEL was performed on the same sections using Terminal deoxynucleotidyl transferase (TdT) to label blunt ends of double stranded DNA breaks. The end-labeling method TUNEL (TdT-mediated X-dUTP nick end labeling) enables the highly sensitive detection of apoptosis in tissue (In Situ Cell Death Detection Kit).

Image Analysis

Images shown are representative of at least 3 rats which gave similar results. At least 10 images were taken at the same magnification from all immunostained probes. Immunostained sections were examined on a Zeiss LSM780. Microscopy images were processed using ZEN2 image processing software.

Statistics

Results are expressed as mean ± SEMs (n = 6). Statistical analysis was performed using PRISM GRAPHPAD version 5.01. One-way analysis of variance (ANOVA) was used to compare multiple data sets and when the p value obtained from ANOVA was significant, Tukey's test was applied to test for differences among groups. Significance was considered to be $p < 0.05$.

Results

Preparation and characterization of nCUR

The nCUR preparation process has been thoroughly optimized over the years in our laboratory (Grama et al., 2013; Shaikh, Ankola, Beniwal, Singh & Kumar, 2009). The preparation used in this study has a particle size of ~300 nm with ~100 μg CUR/mg polymer (Supplement Fig. S1). These freeze dried preparations are rendered easy to reconstitute product with comparable characteristics to the fresh preparation in terms of size and CUR content in the particles. We have established shelf-storage stability of nCUR in our prior studies (Grama et al., 2013).

In vitro testing of nCUR in rat pancreatic β-insulinoma cells (RIN-m5F)

Rat (RIN-m5F) insulinoma cells are widely used *in vitro* model for pancreatic β-cells (Skelin, Rupnik & Cencic, 2010), to study effects of STZ-induced cellular stress and apoptosis. Herein, we have used this model to test the ability of CUR and nCUR to protect STZ induced apoptosis. Untreated or RIN-m5F cells pretreated with CUR or nCUR for 1h were exposed to STZ for 24 h, and viability was assessed using PI assay, which stains dead cells. Treatment with STZ significantly reduced cell viability whereas, CUR and nCUR increased the viability of STZ-treated RIN-m5F cells (Fig. 1a). Similar effects of CUR and nCUR were observed on preventing caspase 8 activation, thereby preventing cell death and improving cell-survival (Fig. 1b).

Multiplex immunoassay for cytokines

Activated macrophages produce proinflammatory cytokines that are involved in up-regulation of inflammatory reactions, promoting apoptosis. Therefore, we measured

cytokine panel of 14 markers that include IL-1 alpha, G-CSF, IL-10, IL-17A, IL-1 beta, IL-6, TNF alpha, IL-4, GM-CSF, IFN gamma, IL-2, IL-5, IL-13, IL-12p70, in plasma and pancreas. STZ insult led to a significant increase in all the 14 markers studied. The nCUR was able to prevent the STZ-induced rise in G-CSF/GM-CSF ($p<0.05$), whereas CUR failed in the case of G-CSF. Both CUR and nCUR were successful in normalizing the proinflammatory cytokines in pancreas tissue homogenates (Fig. 2). However, the plasma analysis revealed no significant differences between controls, STZ or treatment groups for the studied duration [72h] (Supplement Table S2).

Effect of CUR on blood glucose and plasma insulin level

The selective pancreatic beta cell apoptosis caused by STZ challenge is expected to increase blood glucose levels, and a protection from β-cell death should in principle have better glucose control. The nCUR, but not CUR, showed significant ($p<0.05$) reduction in blood glucose in nonfasted rats by 32% and 37% for nCUR10 and nCUR50, respectively, at 72h time point. The reduction in glucose levels also reflects in slight increase in plasma insulin levels, owing to improved ß-cell function (Fig. 3a). Interestingly, statistically significant differences were found in the glucose levels at 48 and 72 h, suggesting possible ß-cell regeneration. However, more detailed experiments on repeated administration are needed for both pre-and post-STZ challenges.

Effect of CUR on pancreatic islets/β-cells

Inflammation is associated with cell death and the ability to effectively target inflammatory pathways will prevent cell death (Santin & Eizirik, 2013) (Carrington, Kos, Zhan, Krishnamurthy & Allison, 2011). The ability of CUR/nCUR to prevent STZ induced

β-cell death was examined by TUNEL assay. The qualitative data clearly suggests that nCUR offered better protection than plain CUR treatment (Fig. 3b), and this was corroborated with plasma glucose levels (Fig. 3a). Immunostaining of insulin is shown in Supplement Fig. S2.

The results of histological analysis of pancreas show well defined islets of Langerhans with intact exocrine of pancreatic tissues in normal control, which is lost in STZ control. Though there was as an improvement in both CUR and nCUR treated groups, the differences were statistically insignificant within (Fig. 4a).

Histiocytes were identified by ionized calcium binding adaptor molecule 1 (Iba1), which is also called allograft inflammatory factor 1 (AIF-1). The immunohistochemical staining of Iba1 showed that the number of infiltrating histiocytes was significantly reduced with the CUR and nCUR treatments ($p < 0.05$) (Fig. 4b).

Under hyperglycemic condition, generation of reactive oxygen intermediates and apoptosis is expected to increase. Therefore, oxidative DNA damage was identified by immunostaining of 8-oxo-dG. A higher intensity of 8-oxodG was observed in diabetic pancreas, compared to control, while, nCUR was able to prevent the damage and CUR had little effect (Fig. 4c).

Safety of CUR and nCUR on sub-acute dosing

From a regulatory standpoint it is important to understand that the carrier systems do not cause any deleterious effects on the biological system. Repeated administration (daily for 28 days) of CUR at 500 mg/kg or nCUR at 25, 50 and 100 mg/kg or the CUR void nanosystems equivalent to 100 mg/kg CUR had no noticeable effects on

inflammatory/oxidative stress, antioxidant enzyme levels or hematological parameters (Supplement Tables S3-S7). At 100 mg/kg in nCUR group showed significant increase in plasma levels as opposed to other groups (Supplement Table S8). Further, no noticeable changes were observed in histologic evaluation of examined rat tissues, including small intestine, spleen, lung, kidney, heart, testis etc. (Supplement Fig. S3).

Discussion

To the best of our knowledge, ours is the first study to demonstrate the ability of nCUR to prevent inflammation and β-cell apoptosis. In doing so, we have also established the safety of the nanosystems, which is critical for clinical translation.

Reformulating drugs or drug-like compounds to address stability, pharmacokinetic (PK)/pharmacodynamic (PD) issues is a common strategy and biodegradable nanosystems are hailed as breakthrough technology for this purpose (Bhardwaj et al., 2005). Despite the huge, largely untapped therapeutic benefit of CUR, a natural anti-inflammatory, numerous clinical trials have failed owing to poor oral bioavailability leading to reduced enthusiasm for CUR therapy (Kumar, 2012; Ratnam, Ankola, Bhardwaj, Sahana & Kumar, 2006; Ratnam, Wadsworth & Kumar, 2011). Our laboratory has conducted a series of investigations on improving CUR stability and oral bioavailability by encapsulating CUR into biodegradable nanosystems that led to improved efficacy in diabetes models (Shaikh, Ankola, Beniwal, Singh & Kumar, 2009; Grama et al., 2013; Grama, Venkatpurwar, Lamprou & Kumar, 2013; Ratnam, Wadsworth & Kumar, 2011).

The current study describes the use of CUR nanosystems (nCUR) for preventing β-cell apoptosis as a possible intervention for diabetes prevention and management.

The nCUR used in this study have a particle size of ~300 nm with ~100 μg CUR/mg polymer. Though there is no clear cut off size for oral delivery, it is generally believed smaller particle size is better absorbed (Ratnam, Bhardwaj & Kumar, 2013). Drug loading is critical for developing translatable dosage forms as initial loading and entrapment will influence the bio-distribution, safety and efficacy (Bhardwaj et al., 2005). This becomes really important for chronic conditions such as diabetes, in that, better drug loads will minimize excess use of excipients. According to USFDA, for conventional formulations, conversion of animal doses to Human Equivalent Doses (HED) based on body surface area requires animal dose in mg/kg to be divided by 6.2 or multiplied by 0.16 to get HED in mg/kg. Based on these assumptions the HED of the dose used in this study will be 480 mg for a 60 kg human with ~4.8 g of associated polymer. Similar doses (360 mg/day, Trial #NCT02104752) of CUR nanosystems made of gum ghatti are currently in use clinical setting. Based on the preclinical studies in rodents, polymer nanosystems developed in the current study may not require daily administration; however this needs to be tested in higher animals or Phase 1 studies to better understand HED conversion of unconventional delivery systems.

Effective prevention or management of diabetes requires the preservation of $\beta-$cells. A large body of data suggests that oxidative stress plays a key role in the development of diabetes and its complications (Acharya & Ghaskadbi, 2010). The weak defense status of islets combined with inefficiency in repairing oxidative DNA damage as compared to other tissues renders the pancreas vulnerable to oxidative stress leading to pancreatic β-cell death. STZ is a toxin that leads to oxidative stress/inflammation and apoptosis of β-cells in the pancreas mimicking autoimmune

diabetes. In the present study, we used RINm5F cell line as a model of pancreatic β-cells, to investigate CUR and nCUR's ability to prevent STZ induced apoptosis. In general nCUR offered better cytoprotective properties compared to unformulated CUR, probably due to intracellular accumulation of nCUR and sustained release of encapsulated CUR (Prokop & Davidson, 2008). The current literature supports the role of caspase-8 in β-cell apoptosis leading to diabetes development (Choi, Schroer, Lu, Cai, Hao & Woo, 2011). The immunofluorescence observations of caspase-8 corroborated with the PI assay. Pre-incubation of RINm5F cells with CUR or nCUR was able to prevent STZ induced cell death that was caused by activation of DNA endonuclease, resulting in apoptotic DNA fragmentation (Kruidering & Evan, 2000).

To determine the protective effects of pretreating rats with CUR or nCUR followed by STZ insult, we examined changes in blood glucose and cytokines levels and correlated them with changes in pancreatic tissue function. We have analyzed granulocyte and granulocyte/macrophage colony stimulating factors (G-CSF and GM-CSF) in plasma and pancreatic tissue homogenates. The cell sources for G-CSF include stroma, endothelium, fibroblasts, monocytes, and macrophages, and for GM-CSF T lymphocytes, endothelium, fibroblasts, monocytes, macrophages and smooth muscle (Root & Dale, 1999). While the plasma levels were not significant between control and STZ groups, a significant increase in pancreatic tissue homogenates of STZ treated rats were observed. The systemic manifestation of inflammation could be duration dependent and such differences were also noticed by other workers (Kirwin, Kanaly, Linke & Edelman, 2009; Maier et al., 2008). In pancreatic tissue homogenates both CUR and nCUR were effective on reducing GM-CSF, while only nCUR was found

to reduce G-CSF. Proinflammatory cytokines play a critical role in the series of events ultimately leading to tissue destruction in diabetes. Among others, proinflammatory cytokines are produced in macrophages and monocytes that triggers the differentiation of T-cells (IL-6) followed by a sequence of events such as, proliferation of cytotoxic T-cells (IL-2), activation of T-cells and macrophages (IL-1, IFN-γ), infiltration of cells into tissues, local inflammation (TNF-α) and local tissue destruction (IL-1) (Wang, Guan & Yang, 2010). The pretreatment with CUR or nCUR led to significant decreases in proinflammatory cytokine production which otherwise were increased in STZ groups. These findings are in agreement with an increase in histiocytic infiltration in STZ groups observed by immunohistochemical staining of Iba1.

The blood glucose levels at 72 h were significantly lower in nCUR groups probably due to enhanced oral bioavailability of nCUR being more pronounced at 50 mg/kg dose. This decrease in glucose was also reflected in a corresponding increase in insulin levels. These findings are in agreement with insulin immunofluorescence, islet numbers and the apoptotic cells determined by TUNEL. TUNEL positive cells in pancreatic islets increased notably in STZ group, while these numbers were dramatically reduced in rats pretreated with nCUR.

It is widely recognized fact that macrophages are involved in the final stage of autoimmune-mediated β-cell destruction, where activated macrophages can directly kill β-cells (Yang, 2008). Oxidative damage is known to correlate well with β-cell apoptosis and glucose levels in diabetes. Further, oxidative stress in diabetes is responsible for a cascade of events such as, stimulation of the polyol pathway, activation of protein kinase C, formation of advanced glycation end products, and subsequent formation of

reactive oxygen radicals. Hyperglycemia, not only generates more reactive oxygen species, but also attenuates anti-oxidative mechanisms by scavenging enzymes and substances. We have examined oxidative DNA damage by 8-oxo-dG immunofluorescence where nCUR offered protection, but not CUR. The results from sub-acute toxicity study suggest nCUR at 100 mg/kg did not cause any deleterious general health effects attributable to the carrier. No significant hematological, histological or biochemical changes were recorded.

In conclusion, this study demonstrates that nCUR is safe and prevents STZ-induced diabetes in rats, at least partly owing to the suppression of oxidative stress, inflammation and pancreatic β-cell apoptosis. Further studies on repeated long-term administration and application to type-2 diabetes prevention will prove beneficial. It would also be interesting to see what insulin combined with nCUR could achieve in both type 1 and 2 diabetes management.

Author contributions

Participated in research design: M. N. V. R. Kumar; R. Ganugula; M. Arora; S. Guo; A. R. Hoffmann; and H. G. Jørgensen.

Conducted experiments: R. Ganugula; M. Arora; and V.P. Venkatpurwar.

Performed data analysis: M. N. V. R. Kumar; R. Ganugula; M. Arora; S. Guo; A. R. Hoffmann; and H. G. Jørgensen; R. Basu; P. Jaisamut; and B. Zhou.

Wrote or contributed to the writing of the manuscript: M. N. V. R. Kumar and R, Ganugula wrote the manuscript and all authors read, revised and approved the manuscript.

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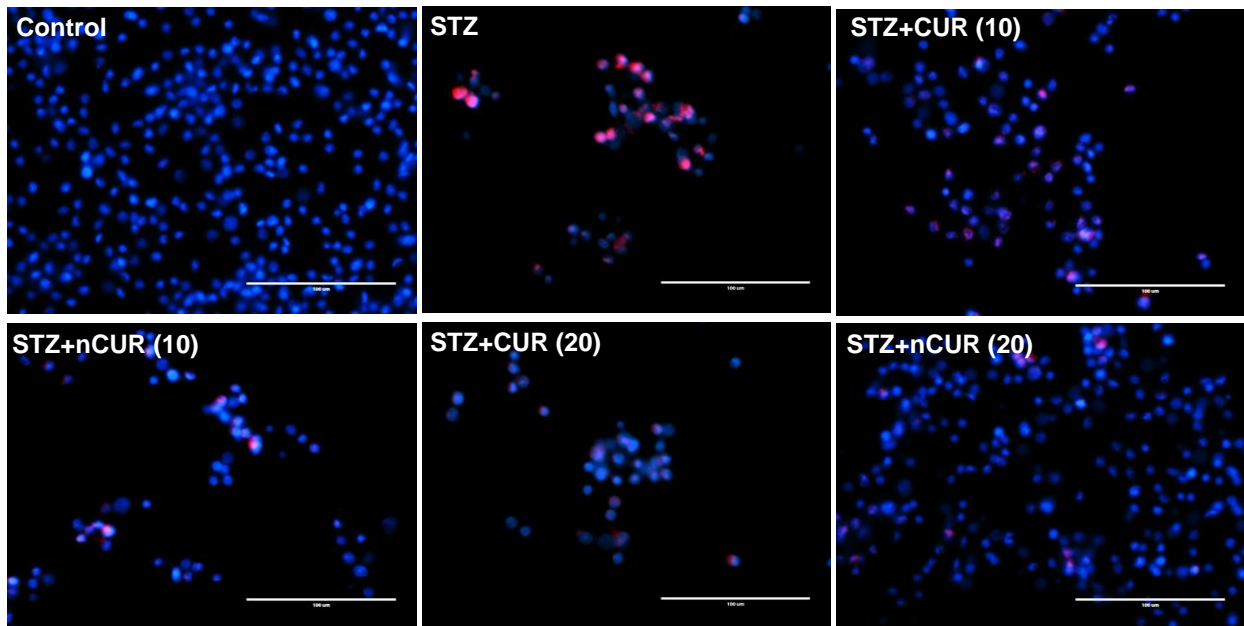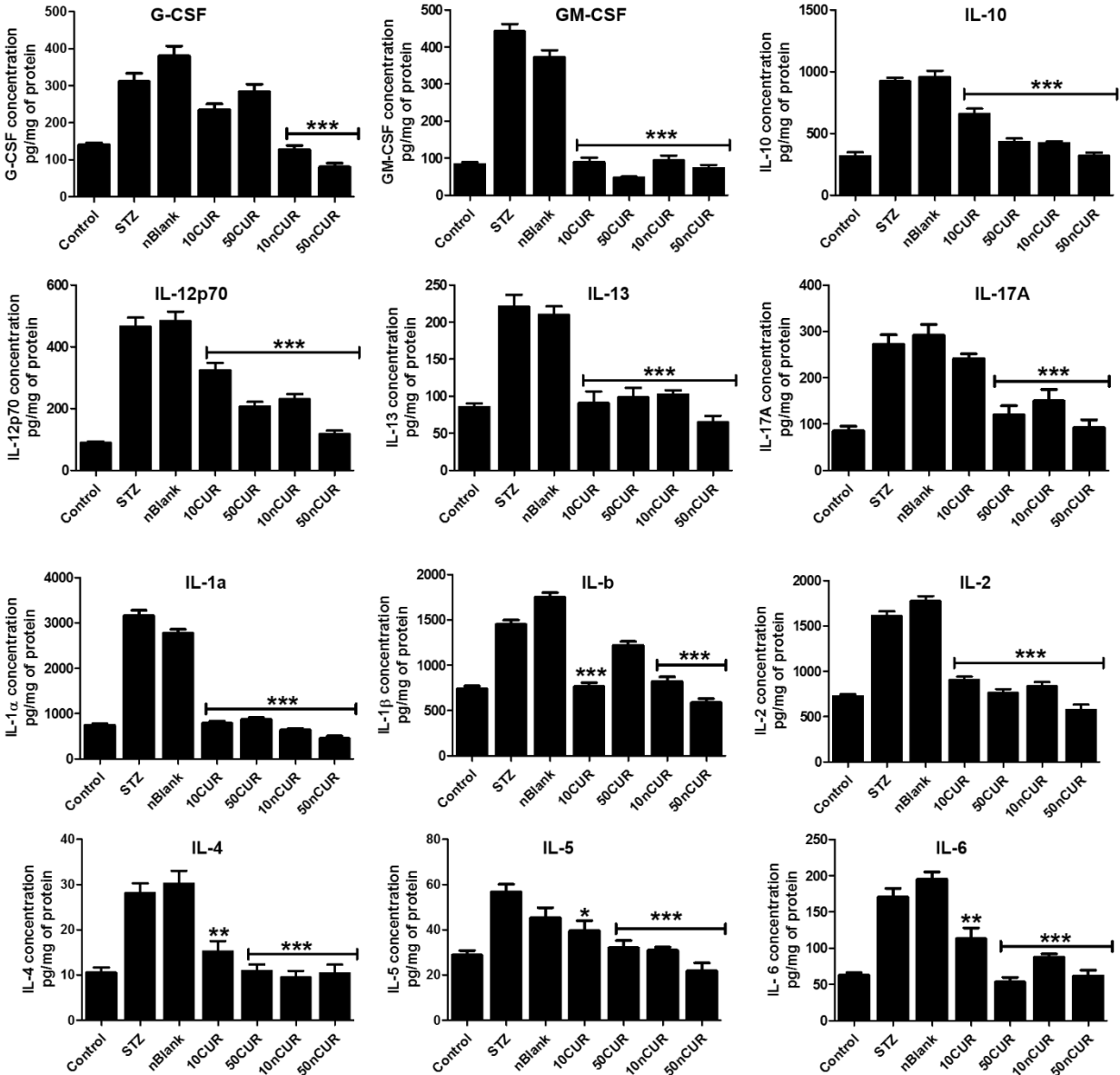
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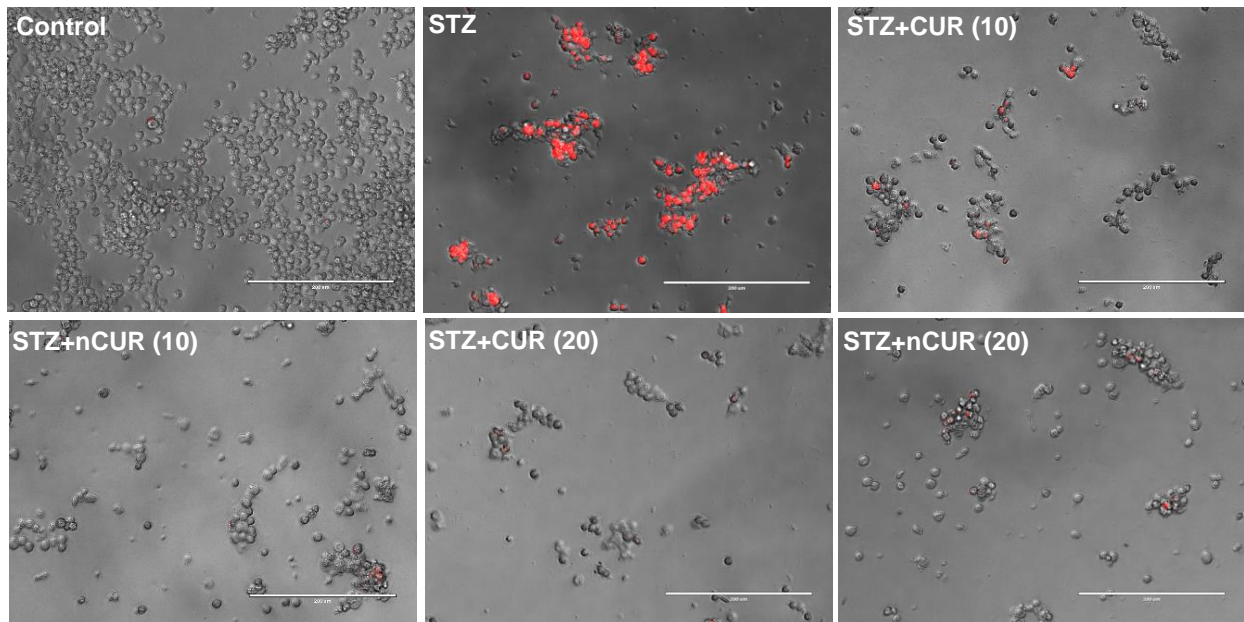
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Figure 1. Pre-treatment with CUR/nCUR prevents STZ induced cell death in RIN-m5f cells. *a)* Cell death was identified by propidium iodide (PI) staining (20x magnification, scale bar represents 200μm). ***b)*** The presence of active caspase-8 was

examined by immunofluorescence using antibodies specific for cleaved caspase-8 (40x

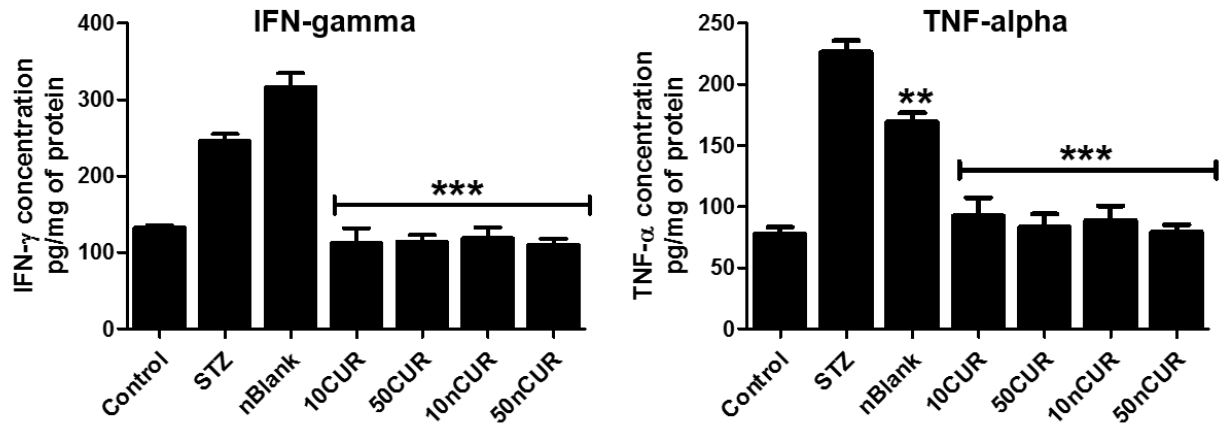magnification, scale bar represents 100μm).

Figure 2. Pre-treatment with CUR/nCUR (10 or 50 mg/kg) orally prior to STZ challenge regulates inflammatory cytokines. Pancreatic tissue homogenates were analyzed 72 h post STZ challenge using multiplex assay. CUR/nCUR regulated the expression of pro- and anti-inflammatory cytokines induced by STZ. Data are presented as mean ± SEM, (n=6) and were analyzed by one-way ANOVA, with Tukey's test. The treatment groups (STZ+10CUR, 50CUR, 10nCUR, 50nCUR) were compared with untreated diabetic controls (STZ) (*$p<0.05$, **$p<0.01$, ***$p<0.001$).
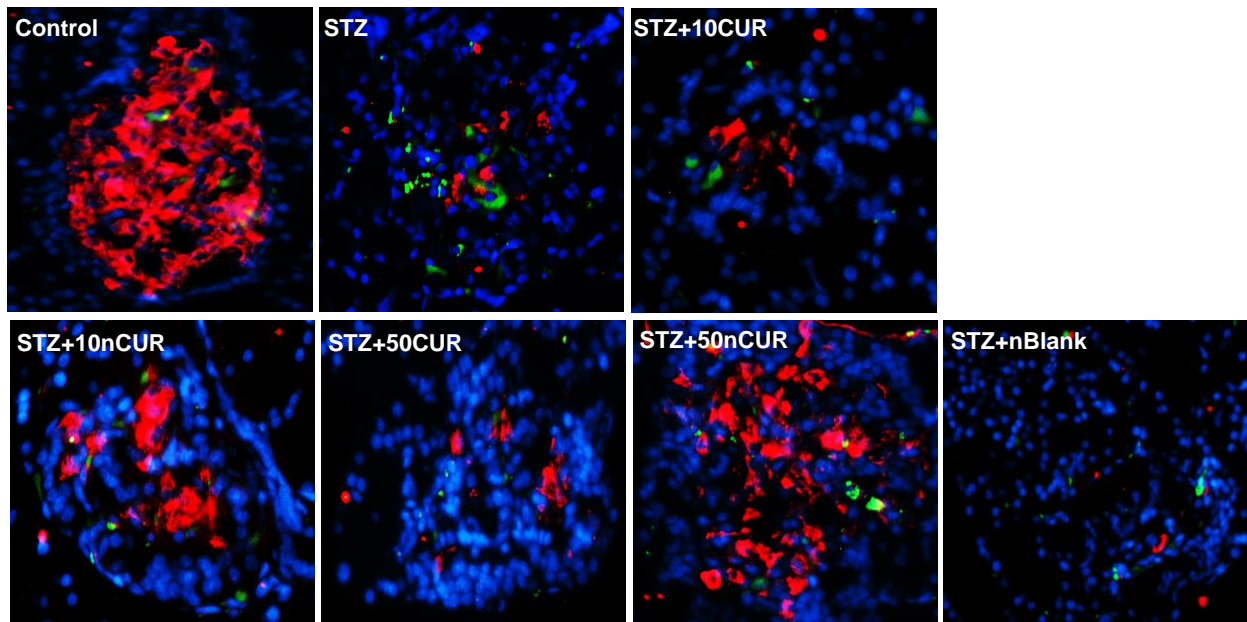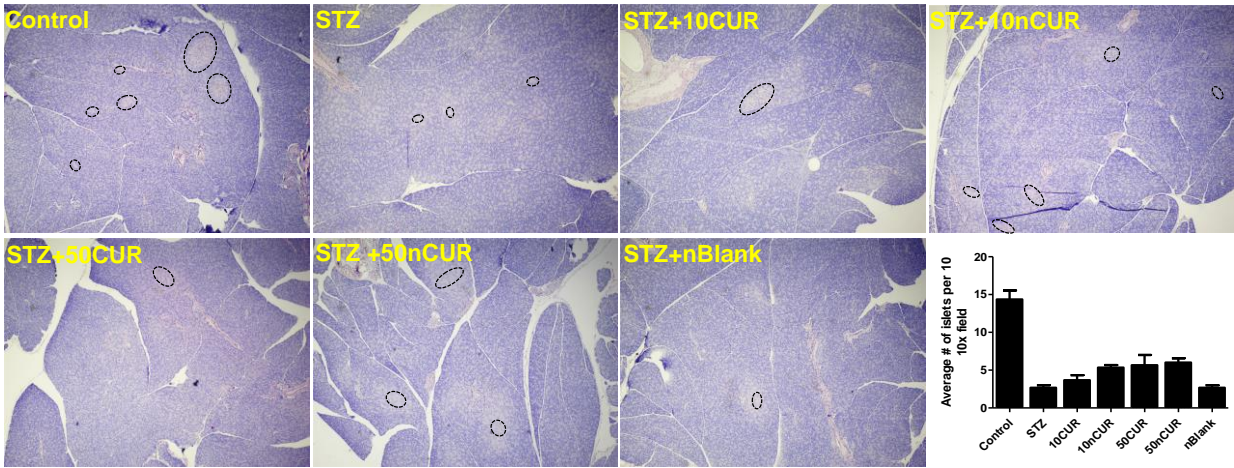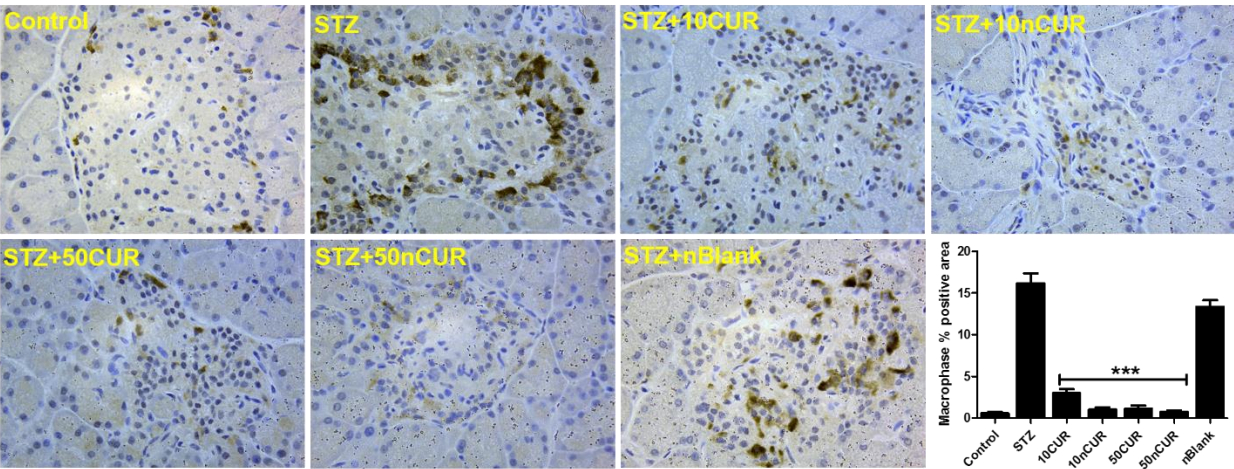
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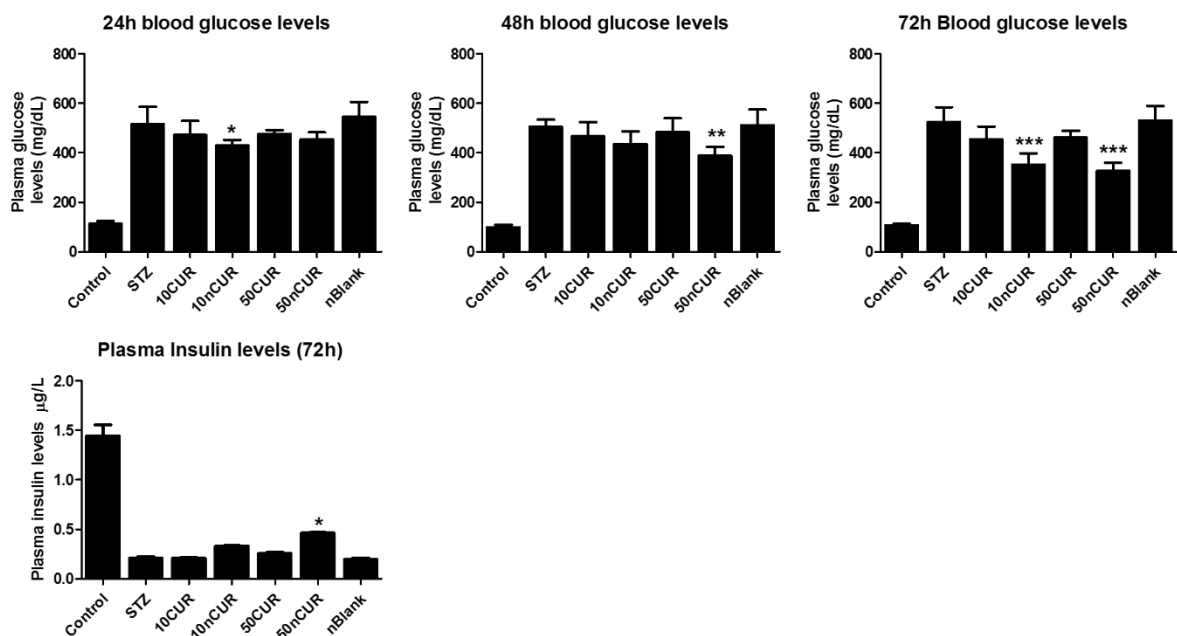
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Figure 3. Pre-treatment with nCUR prior to STZ challenge protects against elevated blood glucose or insulin and loss of pancreatic islet cell numbers through apoptosis. *a)* The blood glucose levels (24h, 48h and 72h) and 72h plasma insulin level post STZ challenge were monitored by glucose strips and ELISA kits (n=6). *b)* Pancreatic islets (72 h) were identified by insulin immunofluorescence (red), and apoptotic cells were determined by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) (green); nuclei were visualized by DAPI staining and shown in blue (n=6). Images were acquired using EVOIS-FL microscope at 40x magnification. Data are presented as the mean ± SEM, and were analyzed by one-way ANOVA, with Tukey's test. The treatment groups (STZ+10CUR, 10nCUR, 50CUR, 50nCUR) were compared with untreated diabetic controls (STZ) (*$P<0.05$, **$P<0.01$, ***$P<0.001$).

a)

Control | STZ | STZ+10CUR | STZ+10nCUR

STZ+50CUR | STZ +50nCUR | STZ+nBlank

b)

Control | STZ | STZ+10CUR | STZ+10nCUR

STZ+50CUR | STZ+50nCUR | STZ+nBlank

c)

Control | STZ | STZ+10CUR

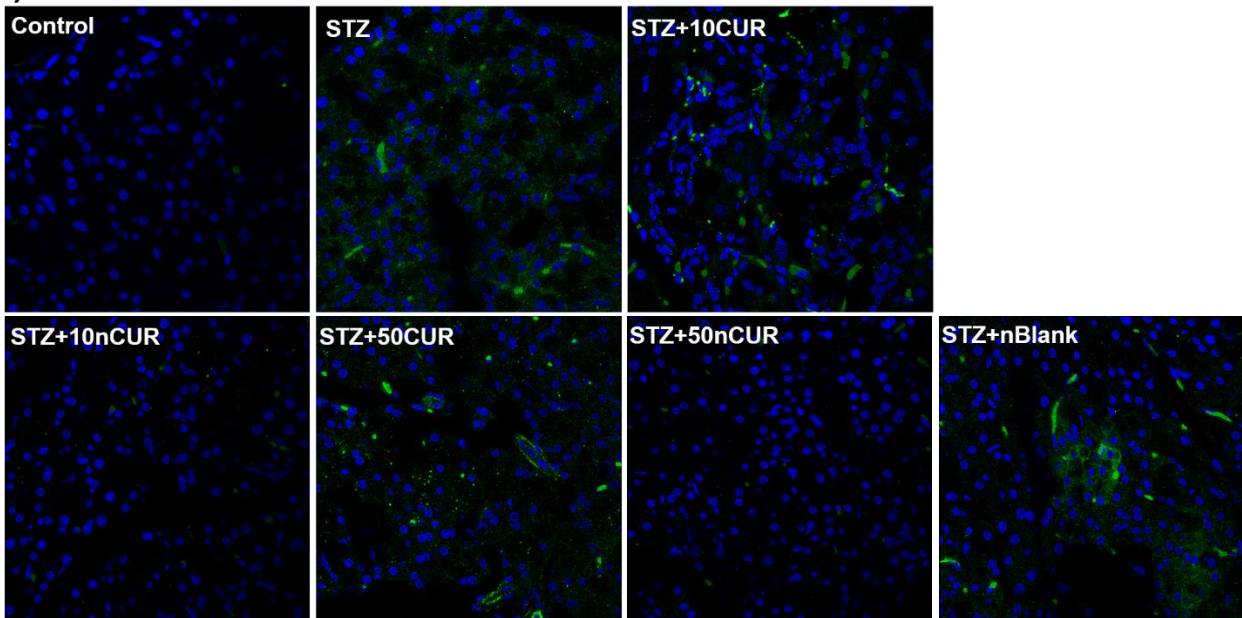STZ+10nCUR | STZ+50CUR | STZ+50nCUR | STZ+nBlank

Figure 4. Pre-treatment with CUR/nCUR prior to STZ challenge supports pancreatic islet cell numbers while reducing histiocytic infiltration and oxidative stress. *a)* Histological changes in pancreatic tissue were observed by hematoxylin and eosin staining. Images were captured using bright field microscope (BFM) at 10x magnification and islets were marked in black dotted ellipses. The average number of islets per field at 10x magnification was plotted, (n=6). ***b)*** Histiocytes were identified by Iba1 immunohistochemistry using DAB (brown) and counterstained with hematoxylin. Images were acquired at 40x magnification using BFM. Histiocytic infiltration as a percentage of field of view area was calculated by NIH-imageJ software. Data are presented as mean ± SEM, (n= 6-12 images from each group) and were analyzed by one-way ANOVA, with Tukey's test. The treatment groups (STZ+10CUR, 50CUR, 10nCUR, 50nCUR) were compared with untreated diabetic controls (STZ) (***$P<0.001$). ***c)*** Oxidative stress was identified by 8-oxo-dG immunofluorescent staining with FITC (green) and nuclei are counterstained with DAPI. Immunostained sections were examined on a Zeiss microscope. Microscopy images were captured using CLSM at 40x original magnification for entire layout. Confocal images were processed using ZEN software.