Effect of kolaviron on islet dynamics in diabetic rats

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**A B S T R A C T**

Kolaviron, a biflavonoid isolated from the edible seeds of *Garcinia kola*, lowers blood glucose in experimental models of diabetes; however, the underlying mechanisms are not yet fully elucidated. The objective of the current study was to assess the effects of kolaviron on islet dynamics in streptozotocin-induced diabetic rats. Using double immunolabeling of glucagon and insulin, we identified insulin-producing beta- and glucagon-producing alpha-cells in the islets of diabetic and control rats and determined the fractional beta-cell area, alpha-cell area and islet number. STZ challenged rats presented with islet hypoplasia and reduced beta-cell area concomitant with an increase in alpha-cell area. Kolaviron restored some islet architecture in diabetic rats through the increased beta-cell area. Overall, kolaviron-treated diabetic rats presented a significant (p < 0.05) increase in the number of large and very large islets compared to diabetic control but no difference in islet number and alpha-cell area. The beta-cell replenishment potential of kolaviron and its overall positive effects on glycemic control suggest that it may be a viable target for diabetes treatment.

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1. Introduction

Diabetes, a disease characterized by hyperglycaemia, increases the risk of developing micro- and macro-vascular complications including atherosclerosis, cardiovascular disease, neuropathy, nephropathy, and retinopathy (Forbes and Cooper, 2013). Blood glucose homeostasis is regulated primarily by two antagonistic hormones: insulin and glucagon produced by beta (beta) - and alpha (alpha) - cells in the pancreatic islets. Insulin is secreted in the fed-state to normalize blood glucose and, glucagon is secreted during a fasted state to raise blood glucose concentrations (Aronoff et al., 2004). Absolute or relative insulin deficiency and/or impaired insulin function contributes to the hyperglycaemic state in type 1 and 2 diabetes. The insulin deficiency in type 1 diabetes is caused primarily by autoimmune responses leading to the infiltration and destruction of pancreatic cells by mononuclear cells (Rothe et al., 1999). In type 2 diabetes, a progressive loss of beta-cells is caused by increased gluc-, lipo- or glucolipotoxicity, endoplasmic reticulum-induced stress, oxidative stress, inflammation (systemic and islet), and beta-cell death (e.g. apoptosis) (Cerf, 2013; Galicia-Garcia et al., 2020). These events converge and contribute and/or exacerbate insulin resistance, beta-cell dysfunction, and failure (Oh, 2015, Lankatillake et al., 2019).

The two main mechanisms for beta-cell replenishment (beta-cell regeneration) are replication (proliferation) of existing beta-cells (beta-cell self-replenishment or beta-cell replication) and differentiation of new beta-cells from non-beta islet cells, pancreatic and extra-pancreatic cells including stem/progenitor cells (i.e. beta-cell neogenesis from non-beta-cells) (Xia et al., 2009, Demeterco et al., 2009, Lyss et al., 2012). Self-replenishment (self-renewal or self-duplication) is the ability of a cell to repeatedly divide without loss of identity or functional potential (Chambers and Smith, 2004). In rodents,
new β-cells are derived mainly from existing β-cells; i.e., β-cell self-replenishment, which is the dominant mechanism for normal β-cell turnover under physiological conditions (Tavana and Zhu, 2011). β-cell populations comprise the individual β-cell (i.e., β-cell numbers) that constitute the β-cell mass in organisms and respond to variable insulin demand governed by physiological and pathological states (Cerf, 2013). β-cell populations are balanced, to a large extent, by β-cell replenishment and death (Cerf, 2013).

Type 1 diabetes is mainly treated with exogenous insulin injection to improve glycemic control (American Diabetes Association, 2011). Management strategies for type 2 diabetes involve administration of insulin alone or with oral or injectable hypoglycemic agents such as: biguanide, thiazolidinediones, SGLT2 inhibitors, GLP-1 agonists and DDP-4 inhibitors. These drugs are also under investigation as adjunctive to insulin therapies for T1D patients (Bacha and Klinepeter Bartz, 2016). Although these drugs have demonstrated benefits in diabetes management, a robust, sustained glycemic control over time has not been achieved and, some associated adverse effects remain unresolved (Borse et al., 2021). Also, non-adherence to the insulin treatment regimen (Doggrell and Chan, 2015), risk of hypoglycemia, low insulin availability and affordability (Li et al., 2019), failure of insulin to achieve glycemic targets (Cohen et al., 2016; Harris et al., 2017), and recent findings of insulin resistance following intensive insulin treatment (Okamoto et al., 2011; Karras et al., 2019) limits the benefits of intensive insulin therapy. Despite the progress in diabetes therapy, maintaining near-normal metabolic control remains a challenge, and the rate of morbidity and mortality from vascular complications is still high (Bertoni et al., 2002; Groop et al., 2018; Lee et al., 2019). Therefore, an imperative need for better glycemic control persists.

The crucial role of the pancreas in glucose homeostasis has prompted investigations targeting the pancreatic β-cell as a promising strategy for treating diabetes. Also, hyperglucagonemia contributes to hyperglycemia by increasing hepatic glucose output. Therefore, α-cells are also potential targets in diabetes (Gaisano et al., 2012, Marroqui et al., 2014). Several natural compounds, including those derived from plants, have received research attention as potential adjuvant or as alternative agents for diabetes management (Yonamine et al., 2016; Shirpoor, 2017; Borse et al., 2021). Some natural compounds and herbs exert regenerating and protective effects on β-cells, thus improving β-cell function and glycemic control. Among these are plant-derived flavonoids (e.g. resveratrol, quercetin, rutin, fisetin and epicatechin), single herbs (Nigella sativa, Artemisia dracunculus L and Vernonia amygdalina) and polyherbal formulations (e.g. Diabecon®, a well-marketed formulation containing herbs and naturally occurring minerals) (Modak et al., 2007; Oh, 2015; Choudhury et al., 2018, Ghorbani et al., 2013). Kolaviron was isolated from these seeds according to the method of Iwu and colleagues (Iwu et al., 1990). Briefly, Fresh seeds of Garcinia kola were purchased from a local market in Ibadan, Oyo State, Nigeria and authenticated by Professor E. A Ayodele at the Department of Botany, University of Ibadan. A voucher specimen (FHI-109777) is available at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan.

2. Material and methods

2.1. Plant materials

Fresh seeds of Garcinia kola were purchased from a local market in Ibadan, Oyo State, Nigeria and authenticated by Professor E. A Ayodele at the Department of Botany, University of Ibadan. A voucher specimen (FHI-109777) is available at the herbarium of the Forestry Research Institute of Nigeria (FRIN), Ibadan.

2.2. Extraction of kolaviron from Garcinia kola seeds

Fresh seeds of Garcinia kola were peeled, sliced and air-dried. Kolaviron was isolated from these seeds according to the method of Iwu and colleagues (Iwu et al., 1990). Briefly, Garcinia kola seeds were grounded to powdered form and extracted with light petroleum ether (bp 40–60 °C) in a soxhlet for 24 hr. The defatted dried product was repackaged and extracted with acetone. The concentrated extract was then diluted twice its volume with water and extracted with ethyl acetate. The resulting concentrate yielded kolaviron, a well-characterized bioflavonoid complex (Ayepola et al., 2013).

2.3. Animals

Forty healthy male Wistar rats (11–12 weeks) weighing 270 ± 25 g (g) were used for the study. The animals were bred at the animal facility of the South African Medical Research Council (SAMRC), with strict adherence to all standard operating procedures. The rats were housed in individual plastic cages at the animal facility of the SAMRC at room temperature (22 ± 2 °C) with 55 ± 5% humidity and an automatically controlled light–dark cycle (12 h/12 h). Standard rat diet (supplied by the SAMRC) and water...
were provided ad-libitum, and rats were acclimatized to the experimental conditions one week before experimentation. The animal study was approved by the Research Ethics Committee of the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology (Ethics number: CPUT/HW-REC 2012/AO4).

2.4. Experimental design and sample collection

At the start of the experiment (before STZ administration), 40 healthy Wistar rats free, from specific pathogens were randomly distributed into four experimental groups (n = 10 per group) using a stratified randomization procedure considering the body weights (~270 ± 25 g) and fasting (~18 h) blood glucose levels (~5.6 ± 1.14 mmol/L). The groups were:

**Group 1:** Non-diabetic control (C)
**Group 2:** Kolaviron-treated non-diabetic control (C + KV),
**Group 3:** Untreated diabetic (D), and
**Group 4:** Kolaviron-treated diabetic (D + KV).

Afterwards, diabetes was induced in overnight fasted rats in groups 3 and 4 by a single intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ; 50 mg kg⁻¹ body weight) in citrate buffer (0.1 M, pH 4.5). Control rats in Groups 1 and 2 were injected with citrate buffer vehicle only. Random blood glucose was measured weekly throughout the study period with a glucometer (Accu-Chek, Roche, Germany) using blood obtained from the caudal vein. Kolaviron, at a dose of 100 mg/kg/day, was dissolved in a vehicle [dimethylsulphoxide (DMSO)] and administered orally (5 days/week; Monday - Friday) for six weeks. Control (C) rats also received vehicles five times a week for six weeks. At the end of the study (6 weeks after confirmation of diabetes in groups D and D + KV), the rats were euthanized with sodium pentobarbital (140 mg/kg) and the pancreas was removed and fixed in 10% (v/v) neutral buffered formalin and embedded in paraffin wax.

2.5. Immunodetection of insulin and glucagon in the pancreas

Fixed pancreatic tissues were cut into 5 μm sections for double immunolabeling of glucagon and insulin. Briefly, each section was de-waxed and immunolabeled for α-cells using a polyclonal glucagon antibody (Dako, Carpinteria, CA) and incubated for 30 min at room temperature. A secondary biotinylated anti-rabbit link antibody (Vector Laboratories, Burlingame, CA, USA) was applied at a 1:1000 dilution, and positive immunolabeling was visualized using the peroxidase diaminobenzidine and substrate chromagen system (Dako Corporation, Carpinteria, CA, USA). Thereafter, β-cells were immunolabeled with a monoclonal insulin antibody (1:10000; Sigma Immunochemicals St. Louis, MO, USA) using the alkaline phosphatase method. This was followed by a separate incubation with a rabbit/mouse link, AP Enzyme Enhancer, and substrate working solution (Envision G/2 System/AP, Rabbit/Mouse Kit). The light microscope was interfaced with a computer via Leica Qwin image analysis software (Leica, Wetzlar, Germany). Stained pancreatic sections were viewed with an X20 objective and images were analysed with an Olympus BX60 light microscope comprised of a mounted Nikon DS-Fi1 digital camera.

2.6. Measurement of α-cell, β-cell area and islet size distribution

The whole section area was measured, and the total islet area was estimated by adding the tissue area measured in each field of view using the interactive measurement option of the Leica software. The total islet area and areas of α-cell and β-cells were determined with the aid of colour segmentation and thresholding on immunofluorescence-stained pancreatic sections. Afterwards, the ratio (%) of the immunoreactive α-cell area and β-cell area to the whole area of islet cells were calculated. The islets were counted and classified as small, medium, large and very large according to the different sizes and numbers of islets in each size group was expressed as a percentage of the total number of islets. All morphometry studies were conducted in a blinded fashion.

2.7. Statistical analysis

The data were expressed as mean values (±SD). Significant differences between glucose levels were determined by two-way repeated analysis of variance (ANOVA) (Treatment × Time as repeated measures) followed by post-hoc Dunnett test for multiple comparisons. Each islet measurement, i.e., total islet number, β-cell and α-cell area, were separately analysed with one-way ANOVA to compare mean differences between groups. Differences were considered significant at p < 0.05.

3. Results

Fig. 2 shows the baseline blood glucose levels in non-diabetic and diabetic rats and glucose levels after treatment with kolaviron. Injection of streptozotocin stimulated a diabetogenic response evident by a significant increase (p < 0.05; about 300 % higher than non-diabetic controls) in blood glucose level by day 5 of STZ injection. The elevation in glucose level was maintained through the study duration. By the 6th week of treatment, kolaviron (100 mg/kg) significantly (p < 0.05) lowered blood glucose level in diabetic rats by ~40 % compared to diabetic controls. Immunohistostaining of the pancreas of normal control rats (C) revealed the peripheral location of α-cells (glucagon) while β-cells (insulin), which are numerous, were centrally located (Fig. 3). Also, the insulin-positive islet area comprised about 90% of the whole islet. However, untreated diabetic rats (D) showed distorted islet architecture (Fig. 3) and a depleted number of islets (Fig. 4a). Islet degeneration post STZ induction was evident by irregularly shaped islets and depleted immunopositive β-cells. Also, most of the β-cells in diabetic rats (Fig. 3; D and D + KV) were very lightly stained compared to non-diabetic rats. On the other hand, the glucagon-
producing \( \alpha \)-cells were strongly expressed and centrally localized in the islets. A decrease in \( \beta \)-cell area and a reciprocally increased \( \alpha \)-cell area were observed in diabetic rats (Fig. 4b and c). In addition, a lower number of large and very large-sized islets in diabetic rats versus control suggest beta cell deterioration (Fig. 5). The staining intensity of immunoreactive \( \beta \)-cells also increased in kolaviron-treated diabetic rats (Fig. 3). As shown in Fig. 4b, injection of STZ caused a \(~43\%\) decrease in \( \beta \)-cell/islet area (35.91 ± 2.68) compared with non-diabetic control rats (61.44 ± 3.18). Kolaviron treatment of diabetic rats resulted in an increased \( \beta \)-cell area (45.83 ± 1.12) compared to control rats (35.91 ± 2.68). Although treatment of diabetic rats with kolaviron did not affect islet number and \( \alpha \)-cell area, the pancreata of kolaviron-treated diabetic rats contained more large islets (in the range of 12,500–20,000 \( \mu m^2 \)) and very large islets (>20,000 \( \mu m^2 \)) and lower numbers of small and medium islets compared to diabetic control rats (Fig. 5).

4. Discussion

Kolaviron administration to diabetic rats lowered blood glucose and showed a potent effect on the islets, as demonstrated by immunohistochemical observations and morphometric results of the islet area. The glucose-lowering effect of kolaviron has been previously reported (Adaramoye and Adeyemi, 2006, Adaramoye, 2012, Ayepola et al., 2013, Tchimene et al., 2016). Furthermore, several mechanisms of the antidiabetic effect of kolaviron have been proposed, including glucose utilization in extrahepatic tissues, direct reduction of macrophage infiltration, the improvement of \( \beta \)-cell function (Ayepola et al., 2013), and increased functional activity of glucose transporters. Previous investigations including studies from our laboratory clarified some mechanistic aspects of the kolaviron beyond its glucose-lowering effect such as antiapoptotic action (Ayepola et al., 2014), the inhibitory effect of liver microsomal glucose-6-phosphatase (Adaramoye and Adeyemi, 2006), anti-inflammatory activity (Ayepola et al., 2013), and antioxidant effect (Oyenihi et al., 2015).

The findings presented herein support the hypothesis on the stimulating action of kolaviron on \( \beta \) cells. Many studies have documented the direct benefits of phytotherapy on the pancreas through different mechanisms, which include: increased islet (i.e. islet hyperplasia) via regeneration of new islets, increased \( \beta \)-cell number and density, reduced lymphocyte infiltration in the islets and reduced oxidative stress indices (Hosseini et al., 2015; Wickramasinghe et al., 2021). A strong correlation exists between the \( \beta \)-cell area and established indexes of \( \beta \)-cell function and glucose control (Meier et al., 2009, Meier et al., 2012). The present findings showed an increase in \( \beta \)-cell area and marked insulin staining in kolaviron-treated diabetic rats despite no change in islet number. The higher number of large islets in kolaviron-treated diabetic rats suggest that the extract could stimulate regeneration or exert protective effects on residual \( \beta \)-cells in STZ challenged rats, thereby improving \( \beta \)-cell function (Hafizur et al., 2015). In kolaviron-treated diabetic rats, there were no effects on \( \alpha \)-cell area or islet number, which suggest that kolaviron is \( \beta \)-cell selective.

Most of the \( \beta \)-cells in diabetic rats were very lightly stained compared to non-diabetic rats - this may reflect low insulin content (Breyton et al., 2014). The increased \( \alpha \)-cell area relative to the islet area suggests an increase in the secretory activity of \( \alpha \)-cells to maintain islet size, a compensatory response to the loss...
of β-cells (Bru-Tari et al., 2019, Zhang et al., 2019). A reduction in β-cell mass would subject β-cells to an increased functional load, which may eventually exhaust insulin release (Leahy, 1990). The ability of kolaviron to improve β-cell degeneration may be due to its stimulating and rejuvenating effects on residual β-cells and other extra-pancreatic action such as modulation of hepatic glucose output and reduced glucotoxicity, modulation of altered cellular redox status, and its anti-inflammatory action (Adaramoye and Adeyemi, 2006, Ayepola et al., 2014, Oyenihi et al., 2015). DNA alkylation and hyperglycemia-mediated oxidative damage by free radicals (reactive oxygen or reactive nitrogen species) have been implicated in β-cell toxicity by STZ (Wu and Yan, 2015). Also, there are reports that hyperglycemia can cause β-cell degeneration by inducing apoptosis (Chang-Chen et al., 2008, Anuradha et al., 2014). The survival of β-cells in kolaviron-treated rats may be partly due to its antioxidant effect (Olayinka et al., 2014), its modulatory effect on the altered inflammatory state (Ayepola et al., 2013, Abarikwu, 2014), and a reduction of β-cell death.

5. Conclusion

The synthesis and release of insulin by the β-cells maintains glucose homeostasis and prevents metabolic diseases. Stressed and inflamed β-cells are functionally compromised and do not effectively respond to increased insulin demand which aggravates β-cell dysfunction resulting in β-cell failure and diabetes (Cerf, 2020). In the present study, we showed that treatment with kolaviron has an ameliorative effect by replenishing the β-cell area. The findings from the present study suggest that kolaviron elicits a direct action on β-cells and enhances β-cell survival. The β-cell replenishment potential of kolaviron and its overall positive effects on glycemic control indicates that it may be a viable target for diabetes treatment.

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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.

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