

Effect of *Citrullus colocynthis* aqueous seed extract on beta cell regeneration and intra-islet vasculature in alloxan induced diabetic male albino rats

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Abstract

Objective: To observe the effect of *Citrullus colocynthis* on beta cell regeneration and intra-islet vasculature.

Methods: This experimental study was conducted at the University of Health Sciences, Lahore, Pakistan, from February 2013 to January 2014. It comprised male wistar rats weighing 100-150g and aged 6-8 weeks. The animals were divided into 6 groups. Group A1 served as control. Diabetes was induced in groups A2, B2 and C2 using single intravenous injection of 50mg/kg of alloxan. Animals having fasting blood glucose >250mg/dl were considered diabetic. Diabetic rats in groups B2 and C2 and their controls B1 and C1 were given 1ml/kg and 2ml/kg of *Citrullus colocynthis* aqueous seed extract orally per day for 14 days. Animals were sacrificed on day 15.

Results: Of the 48 rats, there were 8 (16.7%) in each group. *Citrullus colocynthis* has stabilized the body weight of rats and difference was statistically significant on days 7 ($p < 0.013$) and 14 ($p < 0.001$). *Citrullus colocynthis* significantly reduced ($p < 0.001$) the fasting blood sugar levels in a dose- and time-dependent manner. It increased the islet diameter ($p < 0.001$) and beta cell count ($p < 0.001$). The number of intra-islet capillaries was increased in group C2, but the difference was not statistically significant ($p > 0.05$).

Conclusion: *Citrullus colocynthis* aqueous seed extract stabilised animal body weight and ameliorated hyperglycaemia in a dose- and time-dependent manner which was attributable to regenerative effect on beta cells and intra-islet vasculature.

Keywords: Diabetes, *Citrullus colocynthis*, Oxidative stress, Beta cell regeneration, Endothelial dysfunction. (JPMA 67: 715; 2017)

Introduction

Diabetes mellitus, a debilitating chronic disorder, is posing serious health threats to general population and alarmingly increasing the global disease burden, particularly in the low-income countries.¹ In type 1 diabetes, the environmental and nutritional factors induce oxidative stress and alter immune response by mediating the gene expression of cytokines which thrusts immune mediated beta cell loss.² In type 2 diabetes mellitus, deranged glycaemic control induces glucotoxicity and lipotoxicity exposing the cellular environment to free fatty acid surge which drives beta cell apoptosis and insulin resistance.³ The loss of functional beta cell mass in endocrine pancreas is the ultimate hallmark of tissue injury in both type 1 or type 2 diabetes.⁴

The oxidative stress is the major underlying mechanism in the pathogenesis of diabetes-induced beta cell damage and its devastating complications.⁵ The pathophysiology of progression and complications of diabetes also involves hyperglycaemia-induced endothelial

dysfunction which disturbs the balance of reactive nitrogen and reactive oxygen species (NOx-ROS system).⁶ As diabetes-induced oxidative stress disturbs the pancreatic endocrine-endothelial axis, it seems axiomatic that use of antioxidants can hamper the diabetes-induced oxidative injury and protect beta cells and endothelial cells.⁷

In this milieu, phytotherapy is gaining attention worldwide. *Citrullus colocynthis* (CCT), also known as bitter apple, wild gourd, tumba and wine of Sodom, is known for its medicinal value since long.⁸ The oil yield of *Colocynthis* seeds makes 52% of its contents⁹ and 76.4% of its oil content is composed of linoleic acid.¹⁰ The aqueous seed extract of CCT is known to have many amino acids like Arginine, tryptophan, lysine and leucine; vitamins like biotin, thiamin and niacin; and phytochemical like flavanoids, phenols, alkaloids, tannins, glycosides, triterpenoids and saponins.¹¹

CCT is known to have antioxidant,¹¹ anti-diabetic,^{10,12-14} hypolipidemic,¹⁴ anti-cancer, hepato-protective¹⁵ and anti-inflammatory¹⁶ properties. The present study was designed to observe the role of CCT aqueous seed extract on beta cell regeneration and intra-islet vasculature in alloxan-induced diabetes, hypothesising that it induced both.

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Materials and Methods

This experimental study was conducted at the University of Health Sciences (UHS), Lahore, Pakistan, from February 2013 to January 2014. It comprised male wistar rats weighing 100-150g. The animals were procured from the animal house of the university. Ethical approval was taken from the institutional review board. Declaration of the World Medical Association (WMA) made at Helsinki was followed regarding principles of animal handling in the research.¹⁷ Rats were divided into six equal groups named A1, B1, C1, A2, B2 and C2. Each group was kept in a separate cage and fed with rat chow and water ad libitum. After acclimatisation, diabetes was induced in groups A2, B2 and C2. Group A1 served as normal control and received only saline. Group A2 served as disease control. Groups B2 and C2, and their control groups B1 and C1 were given 1ml and 2ml per kg *Citrullus colocynthis* aqueous seed extract, respectively, through oral gavage once daily for 14 days.

Animals were kept fasted for 12 hours to potentiate the toxic effect of alloxan. Diabetes was induced by giving single intravenous injection of alloxan in the dorsal tail vein. For one rat, 50mg/kg bodyweight per animal alloxan was dissolved in 0.5ml of freshly prepared ice cold saline just before giving alloxan injection in the tail vein of rats, after warming and taping it, as a quick bolus to avoid decay of alloxan due to its short half-life.¹⁸ Alloxan is known to cause fatal hypoglycaemia 4-8 hours after its administration¹⁸ which, in the present study, was prevented by giving 5% glucose solution in water bottles of cages 8 hours after the alloxan injection for overnight. Diabetes was confirmed by checking fasting blood glucose (FBG) of rats after three days, with the help of glucometer (Accu-chek® performa, Roche Diagnostic, Germany). Blood for glucose estimation was taken by pricking the terminal part of tail with the help of lancet after warming and tapping it. Animals having fasting blood glucose more than 250mg/dl, 3 days after alloxan injection, were included in the diabetic groups.

CCT seeds were obtained from a local village of Sindh. Seeds were shade dried and crushed and powdered weighing 200g. Powder was soaked in 400ml of distilled water and stirred with magnetic stirrer for 1 hour at room temperature. Solution was then filtered and filtrate was used to treat experimental animals.¹⁹

Rats in diabetic groups B2 and C2 along with their normal control were given 1ml/kg and 2ml/kg of CCT through oral gavage using feeding tube. Rats were sacrificed on 15th day 24 hours after the last dose. Pancreata were preserved in bouin's solution for 74

hours. Tissues were washed with alcohol to remove picric acid until the yellow tinge of fixative solution turned clear. Tissues were processed in automatic tissue processor and embedded in molten paraffin wax to make tissue blocks for microtomy. Tissue sections of 5µm thickness were obtained to stain with haematoxylin and eosin stain (H&E) and aldehyde fuchsin stain for beta cells and immunohistochemical (IHC) stain using Bandeiraea simplicifolia (BS)-1 lectin to stain intra-islet capillaries.

Weight of the animals (g) and fasting blood glucose (FBG) levels (mg/dl) were recorded on day 1, 7 and 14 of the experiment. Weight of the pancreas (g) was recorded at the end of the experiment. Mean numbers of islets, their diameter and number of beta cells were recorded using micrometry under light microscope. Eight sections were studied per group, at 400 magnifications. The number of islets/mm² was recorded in eight non-overlapping fields using ocular grid.¹⁹

For immunohistochemistry, sections were deparaffinised and hydrated. For antigen retrieval sections were brought to citrate buffer and incubated at 95°C for 40 minutes. Sections were washed 4 times in phosphate buffer saline (PBS). After adding antibody enhancer, sections were incubated for 20 minutes at room temperature and washed 4 times in PBS. Antibody was applied for 30 minutes at room temperature and sections were washed in PBS. Fast red tablet dissolved in 5ml of naphthol phosphate and applied to sections for 10-20 minutes followed by washing in PBS and mounting.

For the quantification of intra islet capillaries, forty eight sections (8 per group) stained immunohistochemically using BS1 lectin were used.²⁰ Microvascular density was observed by counting the number of BS-1 lectin stained capillaries per unit islet area (mm²) in 8 islets in eight randomly selected fields at 400 magnifications.

For counting beta cells, sections stained with modified aldehyde fuchsin were used. Eight randomly selected islets per section (64 islets per group) were studied to count beta cells (no/1000µm²) using point counting method.²¹ Beta cell diameter was determined by measuring the average of 5 inter-nuclear distances, including one nucleus, in five randomly selected islets per section. Inter-nuclear distances were calculated with the help of linear graticule. The number of divisions was counted on linear graticule mounted on ocular micrometer and was multiplied with calibration factor. Mean value was calculated for five randomly selected islets per section.²² Diameter of islets was determined using linear graticule. Major diameter (a) and minor

diameter (b), at right angle to major, was recorded and profile diameter of islet was determined by taking square root of the product of a and b diameters.²³

Data was analysed using SPSS 20. For quantitative parameters, one-way analysis of variance (ANOVA) was applied to calculate the mean values and standard deviation. Post-hoc Tukey test was applied to see which group means differs. Repeated measure ANOVA was used to see the significance of change in parameters that were repeatedly measured (body weight and FBS) at three levels (day 1, day 7 and day 14) in the experiment. Pearson correlation analysis was done to see the relationship between beta cells regeneration and intra-islet capillaries.

Results

Of the 48 rats, there were 8(16.7%) animals in each group. The body weight of rats was significantly decreased in diabetic animals ($p < 0.05$). The difference in body weight, among the groups, was not significant on day 1 of experiment ($p > 0.05$), but it was statistically significant on day 7 ($p = 0.013$) and day 14 ($p = 0.001$). On day 14, weight of rats stabilised with higher dose of CCT treatment (Figure-1).

Repeated measure ANOVA showed that the difference in the body weight of rats within groups at day 1, day 7 and day 14 was significant in groups A1 ($p = 0.000$), A2 ($p = 0.001$), B1 ($p = 0.024$), B2 ($p = 0.000$) and C1 ($p = 0.004$). However, the difference was insignificant in group C2 ($p = 0.064$).

In disease control A2 group, pancreatic tissue weight significantly decreased and difference was significant when compared with controls ($p = 0.001$) and difference was statistically insignificant when A2 was compared with groups B2 and C2 (Table).

Morphometric analysis showed that the number of islets/ mm^2 decreased ($p = 0.001$) in alloxan-induced damage to the islet tissue and the difference was statistically significant when group C2 was compared with control groups ($p < 0.001$); however, group C2 was not

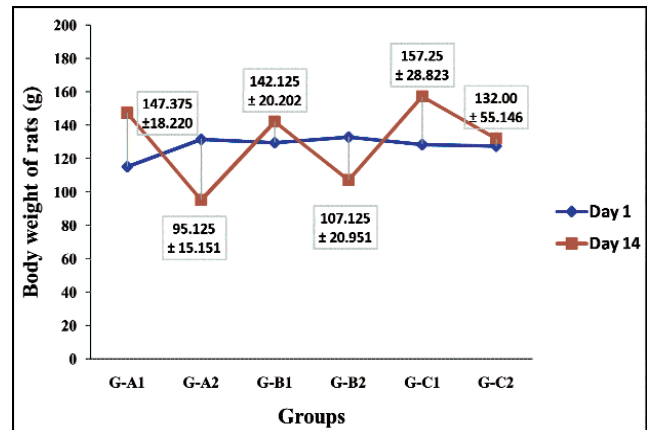


Figure-1: Line graph showing the mean body weight of rats on day 1 and day 14 of Experiment.

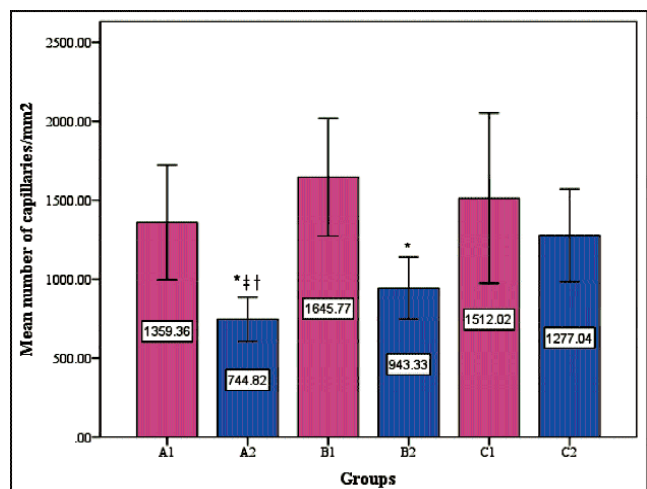


Figure-2: Bar chart showing mean numbers of capillaries per mm^2 . Standard deviation bars are shown. †p-value < 0.05 when compared with A1; *p-value < 0.05 when compared with B1; ‡p-value < 0.05 when compared with C1.

statistically different from groups B2 and A2 ($p > 0.05$).

The difference in the islet diameter among the groups was

Table: Showing comparison of histological parameters among the groups.

Parameter	G-A1 Mean±SD	G-A2 Mean±SD	G-B1 Mean±SD	G-B2 Mean±SD	G-C1 Mean±SD	G-C2 Mean±SD	p-value
Pancreatic wt.	0.65±0.05	0.31±0.04	0.61±0.06	0.31±0.06	0.63±0.05	0.39±0.12	<0.001*
Islet/ mm^2	7.01±1.23	3.47±2.18	8.36±2.02	2.40±1.71	6.97±2.13	2.72±0.80	<0.001*
Islet Diameter	132.71 ± 18.09	103.65 ± 20.27	131.41 ± 21.36	98.58 ± 13.51	116.86 ± 7.57	123.27 ± 22.71	0.001*
B cells/1000 μm^2	3.34±0.66	0.50±0.31	4.08±0.57	1.18±0.94	4.58±0.59	2.75±1.16	<0.001*
B cell Diameter	9.63±1.22	9.51±1.23	9.86±1.55	9.65±1.09	8.80±0.89	10.03±0.33	0.352

SD: Standard deviation

*P ≤ 0.05 was considered statistically significant.

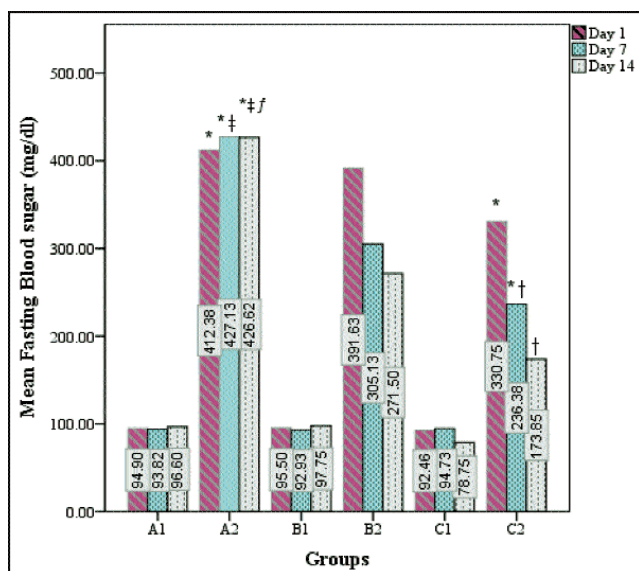


Figure-3: Bar graph showing the comparison of mean values of fasting blood sugar levels on day 1, day 7 and day 14 of experiment. *p-value<0.05 when compared with groups A1, B1 and C1; †p-value<0.05 when compared with group A2; ‡p-value<0.05 when compare with group B2; f-p-value<0.05 when compared with group C2.

significant ($p=0.001$). Islet diameter was decreased in group (A2) and increased after treatment with CCT extract. The difference was significant when groups A2 and B2 were compared with control groups A1 and B1 ($p<0.05$ each); whereas, group C2 was not statistically different from groups A1, B1 and C1 ($p>0.05$).

The mean numbers of beta cells/ $1,000\mu\text{m}^2$ was significantly different when compared among the groups ($p<0.001$). Beta cell count decreased in alloxan-treated rats and treatment with CCT extract significantly increased the number of beta cells in pancreatic islets in groups B2 and C2 which received CCT extract in low and high dose respectively; however, mean number of beta cells in group B2 was not significantly different from group A2. But beta cell count in group C2, which received high dose of CCT extract, was significantly increased when compared with group A2 ($p<0.001$) and B2 ($p=0.002$) and the difference was statistically insignificant when compared with those of group A1. Beta cell count also increased in control groups B1 and C1, and group C2 was significantly different from B1 ($p=0.014$) and C1 ($p<0.001$) in which normal rats received CCT extract in low and high dose respectively. Moreover, the increase in beta cell number was positively correlated with intra-islet capillaries ($p<0.001$). The number of capillaries/ mm^2 decreased in alloxan-treated rats and increased in alloxan plus CCT-treated rats (Figure-2).

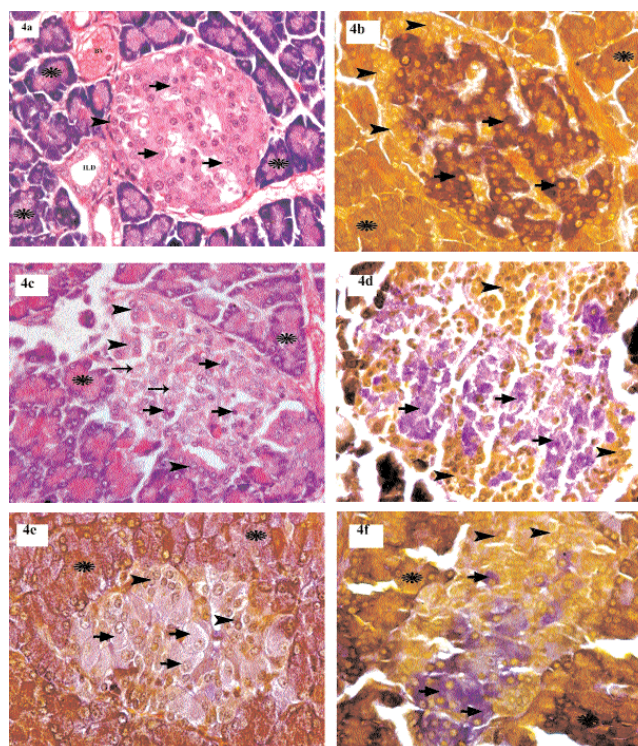


Figure-4: Showing photomicrographs of histological sections of pancreas; (4a&4b) normal architecture of islet tissue showing alpha (arrow heads) and beta cells (arrows) in islet surrounded by acinar tissue in GA1(*); (4c) focal areas of Alloxan induced beta cell necrosis (long arrow) in GA2 (H&E) involving beta cells (short arrows) showing pyknotic and abnormal shaped nuclei with characteristic preservation of cellular architecture of alpha cells arranged at the periphery of islet (arrow heads) surrounded by acinar tissue (*); (4e) reappearance of large sized beta cells (arrows) with intact cellular integrity prominent nuclei and nucleoli as compared to small alpha cells (arrow heads); (4f) Amelioration of beta cell necrosis and re appearance of normal looking beta cells with stainable insulin (arrows) and increased number of alpha cells (arrow heads).

The fasting blood glucose levels were recorded to evaluate the functional integrity of beta cell mass. Mean values of FBS on day 1, 7 and 14 of experiment were significantly different among the groups ($p<0.001$). There was no statistically significant difference among the control groups A1, B1 and C1. However, there was statistically significant dose and time-dependent decrease in mean fasting blood glucose in CCT-treated diabetic groups (Figure-3).

Mean fasting blood glucose levels on day 7 were 427.125 ± 84.021 , 305.125 ± 165.116 , 236.375 ± 131.24 ; whereas, on day 14 they were 426.62 ± 70.96 , 271.5 ± 176.28 , and 173.85 ± 133.58 in alloxan-treated group (A2), alloxan plus low dose CCT-treated group (B2) and alloxan plus high dose CCT-treated (C2) group, respectively.

Repeated measure ANOVA showed that the difference in the FBS of rats within groups at day 1, day 7 and day 14 was insignificant in groups A1 ($p = 0.316$), A2 ($p = 0.211$) and B1 ($p = 0.551$), however, the difference was significant in groups B2 ($p = 0.013$) and C1 ($p = 0.001$) and C2 ($p = 0.002$).

Microscopic examination showed significant necrosis ($p < 0.001$) in alloxan-treated rats specifically involving the beta cells and there was characteristic preservation of cellular architecture of alpha cells arranged at the periphery of islets. Cellular debris of necrotic beta cells appeared as eosinophilic smudges and necrotic changes in nuclei like pyknosis, karyolysis and karyorrhexis were observed. In severe necrosis of islets total disappearance of nuclei was also observed in disease control group. Treatment with CCT extract significantly ameliorated alloxan-induced necrosis (Figure-4).

Discussion

The present study was conducted to observe the effect of CCT on beta cell regeneration and intra islet vasculature in alloxan-induced diabetes. Animal model of diabetes with beta cell loss was developed using alloxan. Single intravenous injection of 50mg/kg alloxan successfully induced diabetes manifested by severe islet necrosis (Figure-4c and d) hyperglycaemia and weight loss in rats and our findings indicated that treatment with CCT ameliorated the alloxan-induced islet injury.

Mean weight of pancreatic tissue (g) was significantly decreased in alloxan-treated rats and treatment with CCT extract did not show any improvement which is in accordance with the finding of Benariba et al.¹³ However, it is assumed that treatment with CCT for a long time may have improved the pancreatic tissue weight.

For the evaluation of regenerative effect of CCT, the numbers and diameter of islets, and number and diameter of beta cells were recorded. Our findings showed significant decrease in the number and diameter of islets in alloxan-treated rats. The number of islets was increased in CCT-treated groups; however, the increase was not statistically significant. The number of small islets comprising of two insulin positive cells or clusters of a few cells were more pronounced in normal control groups who received CCT extract. Small islets were not found in diabetic groups which could be on account of degranulation and lack of stainable insulin in beta cells.

The diameter of islets decreased in diabetic group, a feature, which has been observed in chemically induced animal model of diabetes.²³ Our findings showed significant increase in islet diameter after treatment with

CCT which is in accordance with findings of Jelodar et al.²⁴ who used walnut leaf extract. In our study, the increased islet diameter seemed to be due to proliferation of islet cells, as our results showed significant increase in beta cell count in CCT-treated diabetic rats. This rise in beta cells was dose-dependent, being more pronounced in rats that received CCT in high dose. Many phytochemicals have been reported to cause increase in beta cell mass in chemical-induced animal models of diabetes.²⁵ Previously, the increase in beta cell mass has been linked with CCT oil-rich diet,¹⁰ which was attributed to the growth factor like properties of linoleic component of oil.

The number of beta cells was increased even in normoglycaemic rats in control groups that received CCT. This increase, however, was mild when compared with normal control, but is suggestive of growth promoting effect of CCT on beta cells. It may be on account of insulin-like properties of Citrullus. Guz et al. have documented that exogenous insulin improves glycaemic control and results in beta cell regeneration; however, it was not known that this regenerative effect is due to insulin itself or insulin-induced normoglycaemia.²⁶

Moreover, beta cells also showed hypertrophy (Figure-4e) in CCT-treated diabetic rats, which, however was statistically insignificant, but more pronounced in rats that received high dose of CCT and showed moderate level of fasting blood glucose. The presence of hypertrophied cells is in accordance with a previous study in which hypertrophied beta cells were found in group which received high dose of atorvastatin.²⁷ This suggests that CCT-induced beta cell regenerative mechanisms involve both the hypertrophy and neogenesis of beta cells depending upon the glycaemic status of rats. However, this needs further exploration. It implies that the increase in beta cell mass by neogenesis follows extensive beta cell injury.

Treatment with CCT extract significantly lowered the alloxan-induced hyperglycaemia which is in agreement with previous studies.¹⁰⁻¹⁴ It, very effectively, reduced the blood glucose levels that were within the range of 250-500mg/dl but glucose level, more than 500mg/dl, was not significantly reduced. However, to our knowledge, in none of the previous studies, conducted to see the anti-diabetic effect of CCT, rat models of diabetes with high fasting blood glucose levels, as that of ours, were used.

Previously, anti-diabetic effect of CCT has been attributed to the insulinotropic effect of the amino acid content of CCT seeds.²⁸ Moreover, it has also been linked to the favonoids, particularly the saponin content of Citrullus colocynthis seeds.²⁹ The presence of saponins and their insulinotropic effect has been studied earlier, using

Momordica charantia (bitter melon, or karela) which, like CCT, also belongs to Cucurbitaceae family.²⁵ Moreover, mild hypoglycaemic effect was also observed in control rats who received higher dose of CCT which is in accordance with previous studies.^{12,14}

The present study showed that the increase in number of beta cells was positively correlated with the number of intra-islet capillaries which suggests that endocrine-endothelial axis might have influenced the proliferation or regeneration of beta cells. This finding, although statistically insignificant, in relation with CCT, is being reported for the first time. It is in agreement with a previous study conducted by Marchand et al.²⁷ who documented positive correlation between beta cells and intra islet capillaries, and that the endothelial proliferation precedes beta cell proliferation.

The proliferative effect of CCT on beta cells and intra-islet capillaries might be due to its strong anti-oxidant potential. It is known that diabetes-induced oxidative stress provokes apoptosis of beta cells⁵ and endothelial cells.⁶ In the present study, presumably, ROS might have disturbed the beta cell-endothelial signalling and mutual interaction. It is known that CCT scavenges ROS and replenishes antioxidant enzymes such as catalase, super oxide dismutase and glutathione.¹¹ We suggest that anti-oxidant potential of CCT might have lowered the expression of tumour necrosis factor alpha (TNF α) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B), hence restoring the endocrine-endothelial interaction, signalling the beta cell proliferation. As Ho and Bray reviewed literature and emphasised the protective effect of antioxidants in NF κ B (redox sensitive transcription factor) induced beta cell apoptosis.³⁰ Moreover, CCT is known to decrease the levels of TNF α ,¹⁶ a proinflammatory cytokine. However, rise in beta cell count even in the control groups suggests the growth factor like properties of CCT. In this context, Sebbagh et al.¹⁰ have proposed the Keratinocyte growth factor (KGF) like action of linoleic acid content of CCT seed oil. However, further research, to explore the role of CCT on beta cell regeneration, mediated by KGF, NF κ B and TNF α is required.

As far as the safety index of CCT is concerned, it is found to have no deleterious effect when given in control groups which showed no wasting, change in behaviour or any other discernible sign of toxicity. The seed extracts of CCT are considered safer as compared to other parts of the plant, particularly the pulp which is known to irritate mucous lining of intestine.³¹

The study had its limitations as well. The duration of this

study was short and the safety profile of the higher dose used in the current investigation regarding its toxic effect on the organs over the longer period of time was not evaluated. Although seed extracts are considered safer. However, owing to substantial medicinal virtues of CCT, its dose and time-dependent safety profile need to be evaluated further.

Conclusion

CCT extract was found to be a potent anti-diabetic and has regenerative effect on beta cells in positive correlation with intra-islet vasculature in alloxan-induced diabetes.

Disclaimer: None.

Conflict of Interest: None.

Source of Funding: The financial support by UHS is gratefully acknowledged.

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