CARCINOSIN 30 C PROTECTS ISLETS AGAINST STZ INDUCED BETA CELL DYSFUNCTIONS

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ABSTRACT

Diabetes mellitus is a chronic metabolic syndrome precipitating with several secondary complications. Despite enormous progress in conventional medicine, there is still ambiguity in line of treatment to maintain glucose homeostasis. In this context, homeopathic treatment forms viable and an alternative option in the management of hyperglycemia and has demonstrated beneficial effects in the management of inflammatory diseases including diabetes which reflects state of profound stress and inflammation. The present study addresses the role of Carcinosin 30 c a homeopathic formulation to measure the islet cell function and we have measured cell viability (MTT, LDH leakage), oxidative stress/ inflammatory (ROS, mRNA for TNF-α/and IL-6, Insulin), insulin secretion from Normal and STZ induced diabetic mice. To test our hypothesis, study design included isolation and maintenance of primary islet cell cultures from Normal and STZ mice (>500mg/dl) primary islet cells were divided into four Groups as: A (Control), B (STZ per se), C (Control + Cacinocin 30 c) and D: (STZ per se + Carcinosin 30 c), incubated for 48 hours under optimal conditions in RPMI medium. Our results demonstrated beneficial effects of Carcinosin 30 c against the STZ treated islets and almost reversed cell viability, reduced free radical generation (ROS) and inflammation (TNFα IL-6) with IL-6 being more significant in addition, restored insulin secretion (5.5 and 16.5 mM) compared to STZ per se(Group B). Based the impetus obtained from
the present studies (cytoprotective, insulinotrophic, anti-inflammatory/stress) by Carcinosin 30 c against STZ treatment, its feasible application in the management of diabetes can be explored.

**KEYWORDS**: Carcinosin 30 c, Islets, STZ, cytoprotection, insulinotrophic.

**Abbreviations**

MTT : 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide  
ROS : Reactive oxygen species  
LDH : Lactate dehydrogenase  
TNF α : Tumor necrosis factors  
IL-6 : Interleukin 6  
T1D : Type I diabetes  
T2D : Type II diabetes  
DM : Diabetes mellitus  
KRBH : Krebs Ringer bicarbonate  
STZ : Streptozotocin  
BSA : Bovine serum albumin  
FBS : Fetal bovine serum  
RPMI : Roswell Park Memorial Institute medium  
DCFH-DA : Dichloro-dihydro-fluorescein diacetate  
NAD+ : Nicotinamide adenine dinucleotide  
HEPES : 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

**INTRODUCTION**

Diabetes mellitus (DM) is the most common metabolic disorder characterised by hyperglycaemia that results either from an absolute or relative insulin deficiency (autoimmune responses) depicted in Type I diabetes (T1D) or due to increased peripheral resistance/IR (adipose and muscle) seen in Type II diabetes.\[1,2\] There are several etiological factors associated with DM including changes in life style, food habits, environmental, genetic and more recently epigenetic.\[3\] It has been well documented that DM portrays a state of profound stress and inflammation\[4\] and precipitates towards micro and macro vascular complications under chronic conditions of hyperglycemia or glucose toxicity\[5,6\].

Current practices in managing the glycemic control include: administration of insulin shots\[7\], exercise\[8\], dietary plan\[9\], possibility of stem cell applications\[10\] and several other alternative
approaches.\cite{11} However, the success and efficacy of these approaches on the long term basis needs to be validated despite their benefits in negating hyperglycemia reported in both model systems/and in human scenario.\cite{12} Amongst these, pharmacological interventions have been quite effective, as therapy, nevertheless its limitations have been primarily attributed to the development of resistance and adverse effects advocated for their long term interventions.\cite{13} Interestingly, pancreatic β-cells are highly prone to oxidative stress because they have low expression and activity of antioxidant enzymes, forming the first line of defence system against the generation of free radicals\cite{14} and inflammation.\cite{15,16} Hence, need of the hour is to identify active principles/ factors which not only will elicit cytoprotection to islets and potentiate insulin secretion\cite{17}, vis-a-vis to possess minimal or no toxic effects as a consequence to long term intervention. Carcinosin 30 c, is a potentized homeopathic formulation, derived from cancerous epithelioma of breast tissue and routinely used as a formulation with highest titre efficacy. Carcinosin 30 c has been advocated for patients with family history of cancer, tuberculosis, diabetes and pernicious anaemia, thalassaemia, schizophrenia, arthritis or a combination of all, where the cellular milieu is highly inflammatory.\cite{18,19} Despite its efficacy in clinical parlance to reduce the blood sugar levels in diabetic patients there are no studies undertaken till date to understand its mechanism of action in the management of hyperglycemia. Therefore, present study has been undertaken primarily to understand effects of Carcinosin 30 c on islet cell functions including viability, inflammation, membrane integrity, and insulin secretory responses (basal and stimulation), using primary islet cultures derived from Normal and diabetic (STZ) mice used as the model system.

**MATERIALS AND METHODS**

**Animal experimentation – Experimental Design**

**In vivo**

Experimental procedures were in compliance with the principles of laboratory animal care (NIH publication no. 85–23, revised 1985) and were approved by the Institutional Ethical Committee on Animal (IECA) Experiments, Hyderabad, India. P6F/IAEC/2014/II/VV/SA. The Swiss albino male adult mice / (aged 6–8 weeks old were maintained at the NCLAS Centre, housed in National Institute of Nutrition. The animals were maintained under optimal conditions with temperature 22 ±2°C, relative humidity 50-70%, and with 12 hours of light and dark cycles. The mice were fed with standard diet (chow diet ) prepared in-house and with water *ad libitum.*
The animals were randomly distributed to two groups of six animals each. Group I: Control, Group II: Experimental. Experimental group were injected intra peritoneally a single dose of STZ (200 mg/kg body weight) prepared freshly in chilled citrate buffer pH 4.5 and Controls received the same volume of citrate buffer per se. The blood glucose was measured by tail prick method with a glucometer, and values were expressed in mg/dl (One Touch Horizon, Johnson and Johnson Ltd, USA). At the end of six days post injection both experimental (>500mg/dl) and Control (110-120 mg/dl) mice were sacrificed by CO₂ asphyxiation as per the ethical norms. The effects of Carcinogen 30 c was tested in vitro using the primary islet cell cultures derived from Control and diabetic experimental animals as per the physicians’s recommendation.

In-vitro Assays

Primary Islet cell cultures
Islets were processed from experimental and Control mice under sterile conditions as per our published protocol. Briefly, pancreas were collected under sterile conditions, minced and subjected to Collagenase V (1mg/mL) digestion containing 2mg/mL soy bean trypsin inhibitor (Sigma, USA) and 2% BSA fraction V (Sigma, USA). All steps were performed at optimal conditions of temperature (37 ± 1.5°C) and pH (7.5-7.7). The completion of collagenase digestion was ensured by the turbidity appearance, and the reaction was stopped by the addition of chilled RPMI-1640 containing 10% FBS (1:3 ratio) in order to prevent islet cell death during isolation. The digests were washed three times, and islet-cell-enriched fractions were seeded in RPMI-1640 medium with 10% FBS with antibiotics (Invitrogen, USA). The islets were tested for viability, oxidativestress, cytotoxicity, gene expression and functional responses to assess the effects of Carcinosin 30 c in Controls vs the diabetic (STZ) mice.

Treatment with the Test Compounds (Carcinosin 30 c)
For all the in vitro tests, 250 islets were uniformly counted under the stereo microscope (SMZ-168 Motic) and were used for the studies unless indicated. They were grouped as: A) (Control - , RPMI B) Experimental - STZ per se and C Control + Carcinosin 30 c, D) STZ+Carcinosin 30 c. After incubation for a period of 48 hours, islets from all the Groups (A, B, C and D) were subjected to following assays: viability /MTT (3-(4,5-dimethylthiazol-2-y)l)-2,5-diphenyl tetrazolium bromide), cytotoxicity (LDH), Reactive oxygen species (ROS), Inflammatory and Beta cell specific Gene expression (Insulin, IL-6, TNF-α) and insulin secretion at basal /5.5mMglucose and stimulated /16.5mM glucose respectively.
Viability- MTT assay
After 48 hours of incubation with Carcinosin 30 c (100μl), the cells were centrifuged, and 10μl of MTT reagent was added to cell suspension (A,B,C,D) uniformly taken in in 96 well plate. After 2 hours of incubation at 37°C, acidic isopropanol (100μl) was added and the purplish blue color was measured indicating the formation of formazone at 532nm in spectramax M5. The data has been computed from three independent experiments carried out in duplicates.

Oxidative Stress
The assay was performed similar to our published protocol. Briefly, the cells were treated with 10μM DCFH-DA at 37°C for 30 minutes, and the formation of ROS (fluorescence) was quantitated with excitation at 495nm and emission at 538nm spectramax M5. All values were corrected for the auto fluorescence and fluorescent intensities were normalized by the amount of total Protein in each well. The data has been computed from three independent experiments carried out in duplicates.

Cytotoxic tests (LDH assay)
The LDH is a cytoplasmic enzyme retained by viable cells with intact plasma membranes, but released when there is damage. The LDH catalyses the conversion of lactate to pyruvate upon reduction of NAD$^+$ to NADH/H$^+$; the added tetrazolium salt is then reduced to formazan. All the four samples (A,B,C,D) were processed under identical conditions and LDH assay was performed according to the manufacturer’s instructions (Biovision, K311-400, California, USA). Absorbances were determined in multimode reader using 96 well plate readers at 492nm. (spectramax M5)

Semi-quantitative PCR
Total RNA was isolated, and cDNA was prepared from the samples as per our published protocol. Briefly cDNA was synthesised from 1μg of total RNA at 50°C for 50 minutes using enhanced avian myelo blastosis virus reverse transcriptase (eAMV-RT) enzyme. Each 20-μL reverse transcription reaction mixture contained 1μg of total RNA, 20mM, dNTP, 1mM random hexamers and 100U reverse transcriptase. cDNA was amplified with Sigma AccuTaq DNA polymerase kit according to the manufacturers protocol. Primers were designed with the aid of Primer Quest software (Integrated DNA technologies, Coralville, I A). Semi-quantitative PCR was performed and the amplicons were resolved electrophoretically on 1.2% agarose gels pre-stained with ethidium bromide. The image was captured on a
Bio-Rad Gel Doc system (Bio-Rad laboratories, Hercules, CA, USA) and were quantified using Quantity One D analysis software (Bio-Rad). Results have been expressed as intensities of the band of target gene. β actin was used as the house keeping genes for all the targeted genes described below.

**Table I. Primer sequences and the amplicon size of the genes studied in in vitro studies.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence (Forward 5’-3’/ Reverse 3’-5’)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β Actin</td>
<td>GGCTGTATTCCCCCTCCATCG CAGTTGGTAACAATGCCATGT</td>
<td>154</td>
</tr>
<tr>
<td>Insulin</td>
<td>AACAGCATCTTTGTGGTCCC CACTTGTGGTCCCTCCACTT</td>
<td>110</td>
</tr>
<tr>
<td>IL-6</td>
<td>GTGGCTAAGGACCAAGACCA ATAACGCACTAGGTTTGCCGA</td>
<td>96</td>
</tr>
<tr>
<td>TNF- α</td>
<td>GCAAACCACCAAGCAGAG CGGAGAGGAGCTGACTT</td>
<td>232</td>
</tr>
</tbody>
</table>

**Functional Assay (Insulin Secretion)**

The Islet cell culture (250) from experimental and Controls Groups (A, B,C and D) were subjected to insulin secretion at basal (5.5 mM glucose) followed by high glucose challenge (16.5 mM glucose) as per published method. Briefly, the cells were washed in Krebs Ringer bicarbonate (KRBH) buffer (with 1 mg/ml bovine serum albumin (BSA), 1 mM glucose and 10 mM HEPES). Following this, the cells were incubated at 37°C with KRBH buffer containing 5.5 mM, and then 16.5 mM glucose for 1 hour each. After each incubation, the samples were centrifuged, at RT and supernatants were collected and preserved at -20°C for insulin assay. The insulin secretion was estimated using high range mouse insulin ELISA kit (Mercodia, Uppsala, Sweden) and was expressed as μIU/mg protein. The ratio between 16.5 mM vs 5.5 mM was taken as the fold change in secretion and the values indicated have been from three independent experiments carried out in duplicates for statistical evaluation.

**Statistical analysis**

All data have been represented as mean ± SEM from an average of 6 animals /group. One-way analysis of variance (ANOVA) (Dunnett’s post-hoc test) with Graph-Pad Prism software (San Diego, CA) and *p< 0.05 was considered statistically significant.
RESULTS

Cell viability: MTT Assay
As shown in Fig 1 (A) primary islet cell cultures from Group A, C and D showed viability > 94% as compared to Group B, where STZ treatment per se significantly decreased viability to 33%. Interestingly, when Carcosin 30 c was treated with islets from STZ derived mice (Group D), viability was increased to 3 fold to i.e 93.5% (Group D), unlike that of Controls which did not show any changes (Group C). Values given are mean ± SEM from three independent experiments carried out in duplicates and * p<0.05 denotes significance compared to STZ treated cells.

Fig. 1. Determination of cell viability by MTT assay. As indicated, Carcosin 30 c improved the viability of the primary islet cell cultures derived from diabetic mice unlike Control islets which did not show any change with Carcosin 30 c treatment. *p<0.05 as compared to STZ per se.

Cytotoxicity: LDH release
As shown in the Fig. 2. Normal islets (Group A) had a minimal release of LHD i.e. around 8% of cell toxicity and were similar to Group C (10%) where Carcosin 30 c was added to Normal cells. However, islets derived from STZ treated per se (Group B) or the diabetic group showed a significant release of LDH into the medium accounting for 98% of cell toxicity. Interestingly, the percentage of LDH release was significantly decreased to 10.8% as seen in Group D suggestive of protection towards islet cell integrity of Carcosin 30 c and to negate the STZ induced changes in the islets. Values given are mean ± SEM from three independent experiments carried out in duplicates. *p<0.05 denotes significance compared to STZ treated cells.
FIG. 2 LDH release in primary islet cells after 48 hours incubation Groups A,B,C,and D). As shown, addition of Carcinosin 30 c significantly reduced the LDH leakage of the primary islet cell cultures derived from diabetic mice unlike Control islets which were not changed with Carcinosin 30 c addition. *p<0.05 as compared to STZ per se.

Free radical Measurements: ROS
As demonstrated in Fig 3, ROS levels were comparable between Groups A and C. However, islets derived from the STZ treatment (Group B) showed a significant increase in the ROS levels, and interestingly addition of Carcinosin 30 c to diabetic islets and not to the Controls significantly negated the increase (2.4 fold change). Values given are mean + SEM from three independent experiments, carried out in duplicates. *p<0.05 compared to STZ treated cells.

Fig. 3 Treatment with Carcinosin 30 c to the islet cells derived from STZ treated mice significantly reduced the ROS production (Group D), without much alterations seen in the Controls (Group C). *p<0.05 as compared to STZ per se.
Reverse transcription PCR: Expression for TNF α, IL6 and insulin
Gene expression studies for TNF α, and IL6, markers of inflammation were significantly increased in the STZ treated diabetic mice (Group B). Interestingly, incubation of Carcinosin 30 c with islets from diabetic mice was beneficial and effectively reduced the expression levels of both TNF α/blue, and IL-6/yellow although the fold change with IL-6 reduction was more (Fig.5). Regarding the expression for Insulin (red), STZ treatment which specifically targets the β cells of the pancreas showed decreased expression and addition of Carcinosin 30 c to the STZ islet cells alleviated the insulin expression by 1.4 fold change as indicated. The values given here for Group B and D have been normalized against their respective Controls (Group A and C) accordingly. The data expressed are mean ± SEM of three independent experiments. *p<0.05 normalized against their Controls.

Fig. 4 As shown above, STZ treatment (per se)-Group B showed increased expression of both TNF α/blue, and IL-6/yellow. Interestingly, treatment of Carcinosin 30 c significantly reduced inflammation evidenced in Group D. As depicted (red), the expression of insulin was reduced in the islet cells from STZ per se (Group B), which was corrected with Carcinosin 30 c treatment (Group D). The expression levels have been normalised against their Controls (Group A and C). *p<0.05 normalised against their Controls.

Insulin secretion assay
As shown in Fig 5, Control (Group A) showed maximum insulin secretion at basal (2.7)and with high glucose challenge (5.1). Treatment with Carcinosin 30 c did not have any significant effect on the group A, basal (2.45) and with high glucose(4.75). However, diabetic
group (Group B) showed marked decrease in insulin secretion at basal (1.39) and high glucose (1.95) compared to the Group A. Interestingly, addition of Carcinogen 30 c to STZ group (Group D) showed remarkable increase in the insulin expression at basal (2.5) and high glucose (4.9) level, which corresponds to an increase of 1.7 and 2.5 fold when compared with STZ treated islets. The data were expressed as the mean ± SEM of three independent experiments carried out in triplicate. Significances indicated are shown in comparison to control. *p<0.05.

![Graph](image)

Fig. 5 Demonstrates comparison of glucose stimulated insulin secretion both at basal (5.5 mM) and stimulated (16.5mM) studied in Four groups (A,B,C and D). Addition of Carcinosin 30 c to STZ treated islets (Group D) improved the secretory functions at both basal and with high glucose challenge as compared to STZ (per se) treated islets. However, the insulin secretion was comparable between Group A and Group C. *,# p<0.05; denotes Significance compared to STZ per se group.

DISCUSSION

Management of glycemic control is very critical towards maintenance of glucose homeostasis. There have been several approaches to improve glycemic control which include drug based approaches\(^{[22]}\), insulin shots\(^{[23]}\), insulin mediated stimuli\(^{[24]}\) in recent times possible applications of stem cell therapy. Of great interest in this context are the feasible applications of antioxidants including epicatechin\(^{[25]}\), Vit B6\(^{[25]}\), Vit C\(^{[26]}\), alovera\(^{[27]}\) etc which have been reported to prevent/rescue islets from beta-cell death and damage, to improve glycemic control in diabetes. In similar lines, the impetus obtained from our previous studies advocate for beneficial effects of PLP in vivo and in vitro and ameliorated
the STZ induced pancreatic beta cell dysfunctions. In addition, protection was significant towards islet cell integrity, reduction of inflammatory and oxidative stress responses, insulinotropic and improved the pancreatic beta cell mass\textsuperscript{[28]}. In agreement, we have in the present study demonstrated for islet cell integrity and cytoprotection of the STZ derived islets by Carcinosin 30 c a homeopathic formulation.\textsuperscript{[15]} Substantiating these findings, Carcinosin 30 c was also effective in restoring the LDH activity and indeed studies have well documented for complementary effects of MTT and LDH activity as markers for the cytoprotection of the cells.\textsuperscript{[29]} Viability assessed by MTT assay, requires no labelling of the target cells and provides an estimate of the mitochondrial oxidative process of the living cells.\textsuperscript{[30]} Further, structural membrane damage forms diagnosis of acute hepatitis, myocardial infarction, anaemia and in several transplantation studies where cell viability is of utmost importance and play a crucial role for the efficacy of cell Transplantation.\textsuperscript{[29]} These findings suggest that Carcinosin 30 c rendered protection against the STZ derived islets based on the viability data (Fig 1) and LDH activity/Group D (Fig 2) due to the fact that Carcinosin 30 c had minimal effects when treated with Control islets (group C).

It has been well documented that production of oxygen-free radicals and lipid per oxidation have been the major causative factors noted with the STZ treatment, which enters the cell via the Glut2 transporters causing beta-cell damage.\textsuperscript{[28]} Indeed ROS levels have been well correlated with pathogenesis of several diseases and known to trigger apoptosis, chromatin condensation, and DNA fragmentation in several diseases including T2D.\textsuperscript{[31]} As indicated in Fig 3, beneficial effects of Carcinosin 30 c to alleviate the increased ROS production with STZ treatment has been significant and suggests its scavenging effects (Fig 3) similar to the data reported with several anti-oxidant effects used in the management of DM.\textsuperscript{[32]} Keeping in view of the fact that compared to all the organs, pancreatic β-cells are highly susceptible to stress-mediated cell dysfunction under glucose toxicity, the present observations are significant and can advocate Carcinosin 30 c as β-cell protectant.

It has been well documented that Inflammation form the key intrinsic mechanism in both T1D and T2D\textsuperscript{[33]}. This feature has been well exemplified in the present study where the islets derived from STZ treated mice demonstrated 2.29 fold increase in the expression of inflammatory markers (TNF α and IL-6) and IL-6 being more appreciable than TNF α suggesting for the altered milieu in situ which is in agreement with our data reported from pancreas\textsuperscript{[6]}, adipose\textsuperscript{[34]} and Bone marrow\textsuperscript{[33]} using Obese and T2D model developed
indigenously at our Institute[35] Increase in IL-6 observed could probably help to facilitate increased recruitment of macrophages, which has been well shown as infiltration into the islets under the diabetic conditions.[36] Further, over expression studies in NOD mice have also demonstrated for the interplay of IL-6 with pro inflammatory cytokines (e.g., TNFα) leading to β-cell dysfunction.[36] However, the inciting events that cause systemic inflammation and affecting the metabolic functions could be multifactorial.[37] and not known clearly. Nevertheless, treatment with Carcinosin 30 c to STZ treated islets negated the inflammatory response i.e the decreased the levels of TNFα and IL-6 similar to several other molecules demonstrated as antioxidants.[22,23] To obtain further insights underlining the Carcinosin 30 c effects, we next examined functional response of primary islets incubated with and without Carcinosin 30 c (Group A,B,C and D) by measuring insulin secretion at both basal (5.5 mM) and with glucose challenge (16.5mM). Insulinotrophic effects were seen with Carcinosin 30 c treatment as there was a restoration of the insulin secretion both at basal and with glucose challenge against the STZ insult, (Fig5) vis a vis evidenced by up regulated expression of insulin mRNA (Fig 4/yellow bar). Since normoglycemia is the desired effect of any drug or molecule in the treatment of diabetes we here by show that beneficial effects of Carcinosin 30 c has been note worthy. Probably this is one of the formulation which has demonstrated for the insulinotrophic effects in addition to as scavengers, similar to PLP[28] as well as reported with coriander in clonal β-cell line BRIN-BD1128 showed insulinotrophic effects similar to our present findings with Carcinosin 30 c.[38] Interestingly, addition of Carcinosin 30 c to the Control islet cells (Group C) did not further potentiate the insulin secretion further, unlike our earlier findings with PLP.[28] In addition, regulation rendered by Pdx1[39] Which functions as key transcription factor required for β-development, differentiation, and functions of has also been shown to be regulated by antioxidants.[28] However, in the present study we have not been able to investigate the effects of Carcinosin 30 c on the Pdx-1 regulation. Based on the present findings, we can advocate Carcinosin 30 c as anti-inflammatory, cytoprotective and insulinotrophic, to ameliorate the STZ mediated β cell dysfunction.

**CONCLUSION**

From these findings we advocate for the feasible application of Carcinosin 30 c in the management of frank diabetes based on its cytoprotective, insulinotrophic, anti-inflammatory/stress, combined with insulinotrophic effects. However, further studies are
needed to understand the cellular and molecular site of actions to pin down on its therapeutic functions.

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CONFLICT OF INTEREST
The authors declare no conflict of interest.

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