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Ameliorative effect of chlorophyllinon oxidative stress in experimental model of diabetes

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Abstract

The aim of this present study was to investigate the effect of chlorophyllin (CHL) on oxidative stress in Streptozotocine (STZ) induced diabetic mice.

For the study, mice were divided into Group A: normal control, Group B: diabetic control, Group C: diabetic mice treated with the ascorbic acid, and Group D: diabetic mice treated with CHL. Levels of Reactive Oxygen Species (ROS), lipid peroxidation, protein carbonyl, superoxide dismutase (CuZn SOD &Mn-SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) activities were examined in kidney and heart tissues of different experimental groups. Histological and ultrastructuralstudies were also carried out to evaluate any changes in tissues as well as sub-cellular organelles.

ROS, lipid peroxidation, and protein carbonyl levels have been significantly decreased with concomitant increased of CuZn SOD, Mn-SOD, CAT, GPx, and GR activity in CHLtreated diabetic mice. The histological and ultrastructural studies showed that CHL attenuates the detrimental effect of oxidative stress and alleviated tissue injuries in STZ induced diabetic mice. These results suggested that CHL possesses antioxidative activity and has the potential to amelioratediabetes-associated oxidative stress in mice.

Keywords: Diabetes, Oxidative stress, Streptozotocin, Chlorophyllin, Ascorbic acid

Introduction

Diabetes mellitus (DM) is a serious metabolic disorder with the key manifestation of prolonged hyperglycemia that is caused by either insulin deficiency or insulin inaction or both. The prolonged hyperglycemic condition during DM stimulates the production of more ROS through inducing several mechanisms such as glucose oxidation, polyol pathway, protein kinase C activation, and advanced glycation end product formation [1,2]. Mammalian cells are equipped with both enzymic and non-enzymic antioxidant defense systems to minimize the cellular damage caused by ROS. However, the relative overload of ROS and diminution of antioxidant defense systems activity in DM contributes to the generation of more oxidative stress that plays a crucial role in the pathogenesis and complications of DM [3,4].

Most of the present oral hypoglycemic agents have prominent side effects and fail to significantly alter the course of diabetes. Many reports have suggested that medicinal plants,phytocompounds, and phytocompound-derived chemical substances are effective in the treatment of diabetes [5,6]; produce less or no side effects.Chlorophyllin is a sodium-copper salt of chlorophyll molecule and water-soluble unlike its parent compound. CHL possess therapeutic importance due to its antimutagenic and anticarcinogenic [7], antioxidative [8], and antihyperglycemic effects [9] in different experimental systems. However, the effect of CHL on oxidative stress in the context of DM is unknown; therefore study has been carried out to evaluate the effect of CHL on oxidative stress in STZ-induced diabetic mice.

Materials and Methods

Chemicals

STZ, CHL, 2',7'-Dichlorofluorescein diacetate (DCFH-DA), 2',7'-Dichlorofluorescein (DCF), Thiobarbituric acid (TBA), 1,1,3,3 Tetraethoxypropane (TEP), Pyrogallol, Glutathione reductase (GR) were procured from Sigma-Aldrich Co. (St. Louis, MO, USA). The other chemicals were of analytical grade and procured from Merck Co. (Mumbai, India), Sisco Research Laboratories (Mumbai, India), and Himedia (Mumbai, India).

Experimental animals

Healthy male swiss albino mice (Balb/C strain), 25 – 30 gm of body weight (b.w.), housed in a room maintained at temperature 22^{0}C on a twelve hrs light/dark cycle were used for the study. They were provided a standard laboratory feed and water *ad libitum*. All the

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experiments were carried out in accordance with the Institutional Ethics Committee (IEC) guidelines.

Induction of DM

Diabetes was induced by administering 150 mg/kg b.w. dose of STZ intraperitoneally, prepared in ice-cold citrate buffer (0.1 M, pH 4.5) to overnight starved mice. After injection, mice had free access to food and 5% glucose solution overnight to counter the hypoglycemic shock [10].Mice with fasting blood glucose level 200 mg/dl were selected and used for the experiments.

Experimental design

The male Swiss albino mice were divided into four different groups for the study.
Group A: the normal control group.
Group B: the diabetic control group.
Group C:the diabetic mice treated with the ascorbic acid (50 mg/kg b.w.).
Group D:the diabetic mice treated with CHL (50 mg/kg b.w.).
Group A and Group B were administered only the vehicle (10 ml/kg b.w.). Doses were administered intraperitoneally and alternatively for 28 days.

Isolation of mitochondrial and cytosolic fractions

Mice were sacrificed by decapitation and dissected to extract kidney and heart tissues at the end of the experimental period. Tissues were homogenized to make 10% (w/v) homogenates in ice cold 10 mM HEPES buffer, pH 7.4 containing 0.2 M mannitol, 50 mM sucrose and 1 mM EDTA. Tissue homogenates were centrifuged at 1000g for 10 mins at 4^0C to sediment nuclei and cell debris. The supernatant was collected and centrifuged at 7500g for 10 mins at 4^0C. The resulting mitochondrial pellet was washed by suspending gently in homogenate buffer and resedimented at 7500g for 10 mins at 4^0C to obtain the mitochondrial fraction. The postmitochondrial supernatant was centrifuged further at 15000g for 10 mins at 4^0C and the resulting supernatant was used as cytosolic fraction.

Protein estimation

The protein concentration of the cytosolic and mitochondrial fraction was determined according to the method of Bradford [11]using bovine serum albuminas the standard.

Measurement of ROS production

The generation of ROS was determined by using DCF as a probe, according to the method of LeBel et al. [12] with some modifications. Briefly, 965 µl of phosphate buffer (0.1M, pH 7.4) was taken in a test tube. The assay was initiated by adding 10 µl of 2 µM DCFH-DA dissolved in 1.25 mM of methanol and 25 µl of the sample. The mixture was shaken gently and incubated in the dark for 1 hr at 37^0C. The rate of oxidation of DCFH to DCF was recorded at an excitation wavelength of λ488 nm and an emission wavelength of λ525 nm at 37^0C in a Varian Cary Eclipse Fluorescence Spectrophotometer. The standard curve was prepared by taking various concentration of DCF and the result was expressed as nmol DCF/mg protein.

Lipid peroxidation assay

Lipid peroxidation assay was performed by measuring thiobarbituric acid reactive substances (TBARS) according to the method of Okhawa et al. [13].Briefly, 1.5ml of 20% glacial acetic acid (pH 3.5), 0.2mL of 8.1% Sodium dodecyl sulfate, 1.5mL of 0.8%TBA, 0.7mL of distilled water, and 0.1mL of tissue homogenate or TEP standard were pipetted into test tubes. The tubes were vortexed and then kept in a boiling water bath at 95^0C for 60 mins. After incubation,the final volume was made to 5 mL by adding 0.7 ml of distilled waterto each tube. 5 mL of butanol:pyridine (15:1) mixture was added and the tubes werethen vortexed thoroughly for 2 mins. After centrifugation at 3,000 rpm for 10 mins at 25^0C, 1 ml from the upper organic layer was taken in a cuvette and the absorbance was measured at λ532 nm against an appropriate blank without the sample. The result was expressed as nmolmalondialdehyde (MDA)/mg protein.

Protein carbonyl assay

The protein carbonyl level was determined according to the method of Levine et al. [14]with some modifications. Briefly, thesamplewas incubated with streptomycin sulfate (10% w/v) for 15 mins and centrifuged at 11000g for 10 mins at 4^0C. The supernatant was divided equally into two microcentrifuge tubes; 10 mM2,4-dinitrophenylhydrazine (DNPH) was added in one tube and 2MHCl was added in another tube to use as areferenceblank. The tubes were allowed to stand at room temperature in the dark for 1 hr, with vortexing every 15 mins and then added 20% of TCA to precipitate the proteins. The tubes were centrifuged at 11000g for 10 mins at 4^0C and supernatant were discarded. Protein pellet of each tube was washed carefully with absolute ethanol:ethyl acetate solution (1:1) and centrifuged at 10000g for 10 mins at 4^0C. The pellet was dissolved in guanidine hydrochloride (6M) and absorbance was recorded at λ360 nm. Protein carbonyl level was calculated using molar extinction coefficient of aliphatic hydrazones i.e., 22 x 10^3 M^{-1} cm^{-1}. The result was expressed as nmol protein carbonyl/mg protein.

Enzyme activity assays

The activity of CuZnSOD and Mn-SOD were determined according to the method of Marklund & Marklund [15] with some modifications. The absorbance was measured spectrophotometrically at λ 470 nm. The specific activity was expressed in terms of units/mg protein in which one unit corresponds to the amount of enzyme that inhibited the autoxidation reaction by 50%.

The CATactivity was determined according to the method of Aebi [16] by calculating the rate of degradation of hydrogen peroxide (H_2O_2)at λ240 nm taking 71 $mM^{-1}cm^{-1}$as extinction coefficient. One unit is defined as 1 mmol of H_2O_2 consumed/min and the specific activity was expressed as units/mg protein.

The GPx activity was determined by the method of Paglia& Valentine, [17] as modified by Lawrence and Burk [18]. The absorbance was measured spectrophotometrically at λ340 nm. The extinction coefficient of NADPH i.e. 6.22 $mM^{-1}cm^{-1}$ was used to calculate specific activity and it was expressed as mUnits/mg protein. One unit of GPx activity is defined as 1 µmol of NADPH oxidized per min at 37^0C.

The GR was assayed according to the method of Carlberg&Mannervik [19]. The activity of the enzyme was measured at λ340 nm following the oxidation of NADPH. One unit of GR activity is defined as 1 µmol of NADPH oxidized per min at 37^0C and the specific activity was expressed as units/mg protein.

Histological study

The histological study of kidney andheart tissues were carried out according to the method of Kiernan[20] with slight modifications. Briefly, tissues were first fixed in Bouin's solution for 4 hrsand washed overnight with tap water to remove theyellowcolor of Bouin's solution. Tissues were dehydrated with ethanol gradients(70 – 100%) before embedding in paraffin wax. The paraffin embedded tissues were trimmed into 7 µm sized sections using a rotary microtome. Subsequently, sections were fixed on glass slides, deparaffinized and stained with hematoxylineosin (H&E). Eventually, sections were mounted with DPX and observed under Olympus BX51 light microscope.

Ultrastructural study

Tissue samples were prepared for ultra structural study according to the method of Hayat [21] with some modifications. Briefly, tissues were cut into approximately 1 mm sized pieces and fixed in Karnovsky's fixative. The fixed tissue pieces were washed in 0.1 M cacodylate buffer and centrifuged at 10,000g for 1 min. Tissue pieces were fixed again in 1% osmium tetraoxideand dehydrated with acetone gradients (70% - 95%). Tissue pieces were embedded in a mixture of embedding medium and propylene oxide followed by sectioning with an ultra microtome. The sections were stained with uranyl acetate and viewed under Transmission Electron Microscope (TEM).

Statistical analysis

Results are expressed as mean ± SEM for six mice in each group. One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was performed to compare differences between experimental groups using the statistical package "IBM SPSS Statistics 19.0 for Windows". Statistical significance was set at $p < 0.05$.

Results

Measurement of ROS production

The level of ROS in kidney and heart tissue homogenates of different experimental groups have been represented infigure1. The result showed that ROS level was significantly decreasedin both ascorbic acid-treated diabetic mice of Group C (12.32 ± 0.52nmol DCF/mg protein and 18.67 ± 0.36 in kidney and heart respectively) and CHL-treated diabetic mice of Group D (14.14 ± 0.18 and 22.34 ± 0.53 in kidney and heart respectively) as compared to the diabetic mice of Group B (17.26 ± 0.45, and 26.22 ± 0.32 in kidney and heart) in our present study.

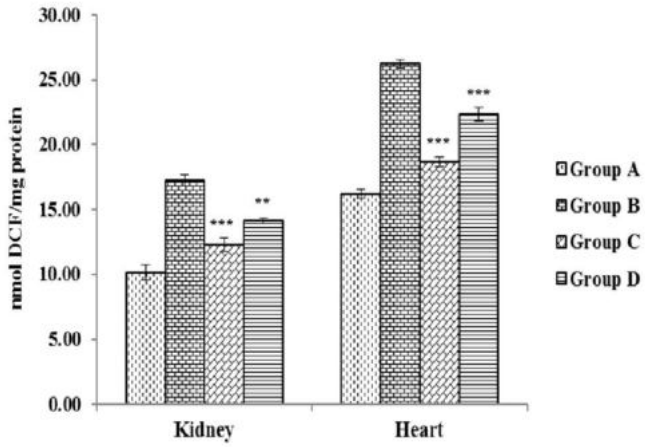

Figure 1. Effect of CHL and ascorbic acid on ROS level in kidney and heart tissues of STZinduced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: $^{**}P < 0.01$ and $^{***}P < 0.001$ as compared to the Group B.

Lipid peroxidation assay

Diabetic mice treated with ascorbic acid of Group C (0.59 ± 0.02nmol MDA/mg protein and 1.55 ± 0.14 in kidney and heart respectively) and CHL of Group D (0.59 ± 0.02 and 1.66 ± 0.12 in kidney and heart respectively) exhibited significant decrease of MDA level when compared to the diabetic mice of Group B (0.72 ± 0.03 and 2.12 ± 0.10 in kidney and heart respectively) as shown the result in figure 2.

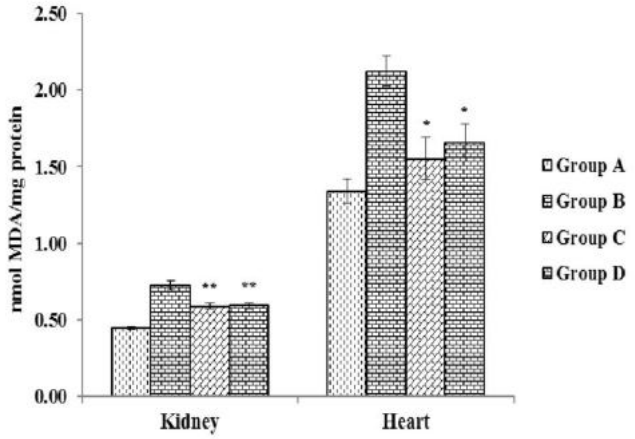

Figure 2.Effect of CHL and ascorbic acid on lipid peroxidation in kidney and heart tissues of STZ-induced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: $^{*}P < 0.05$ and $^{**}P < 0.01$ when compared to the Group B.

Protein carbonyl assay

As shown the result in figure3, the level of protein carbonyl was found significantly higher in reference diabetic control group (3.85 ± 0.18nmol protein carbonyl/mg protein and 5.78 ± 0.12 in kidney and heart respectively) as compared to the normal mice of Group A in the study. The level of protein carbonyl has been significantly decreased in ascorbic acid-treated diabetic mice of Group C (2.61 ± 0.17 and 4.11 ± 0.15 in kidney and heart respectively)and CHL-treated diabetic mice of Group D (2.92 ± 0.08 and 4.17 ± 0.09 in kidney and heart respectively) when compared to the reference diabetic control group in our present study.

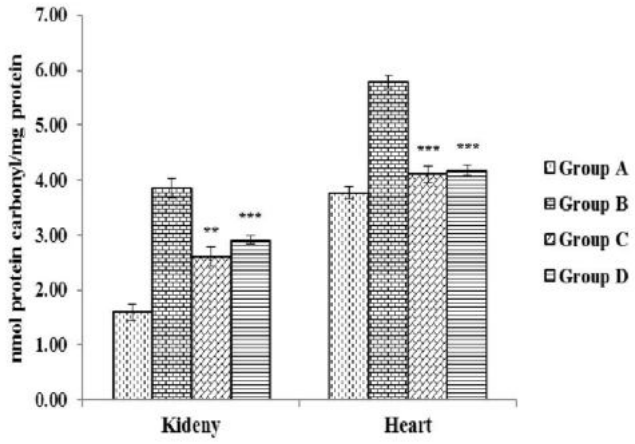

Figure 3. Effect of CHL and ascorbic acid on protein carbonyl level in kidney and heart tissues of STZ-induced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: $^{**}P < 0.01$ and $^{***}P < 0.001$ when compared to the Group B.

Enzyme activity assays

As shown the result in figure 4, Administration of ascorbic acid significantly increased the activity of CuZn SOD in diabetic mice of Group C as compared to the Group B (2.31 ± 0.10vs1.81 ± 0.09 in kidney and 1.85 ± 0.10vs 0.97 ± 0.11 in heart). Similarly, the activity of CuZn SOD was found significantly higher in CHL-treated diabetic mice of Group D as compared to the diabetic mice of Group B (2.18 ± 0.03 vs 1.81 ± 0.09 in kidney and 1.94 ± 0.15vs 0.97 ± 0.11 in heart)in our present study.

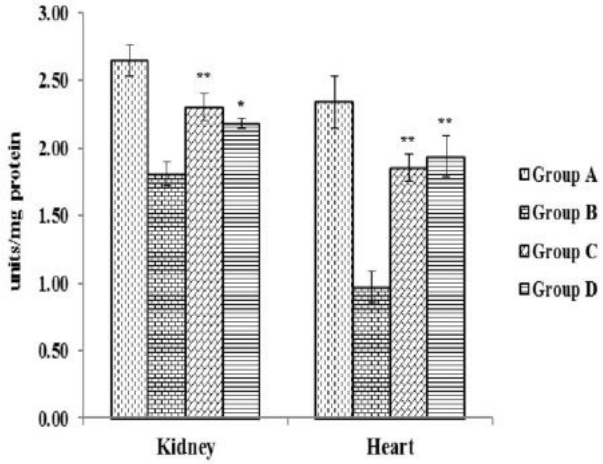

Figure 4. Effect of CHL and ascorbic acid on CuZn SOD activity in kidney and heart tissues of STZ-induced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: $^{*}P < 0.05$ and $^{**}P < 0.01$ as compared to the Group B.

The activity of Mn-SOD in kidney and heart tissue homogenates has been represented in figure 5. The result showed that as compared to the diabetic mice of Group B, the activity of Mn-SOD was significantly higher in both ascorbic acid-treated diabetic mice of Group C (0.68 ± 0.03 vs 0.35 ± 0.01 in kidney and 0.40 ± 0.01 vs 0.25 ± 0.02 in heart) and CHL-treated diabetic mice of Group D (0.65 ± 0.03 vs 0.35 ± 0.01 in kidney and 0.34 ± 0.02 vs 0.25 ± 0.02 in heart) in our present study.

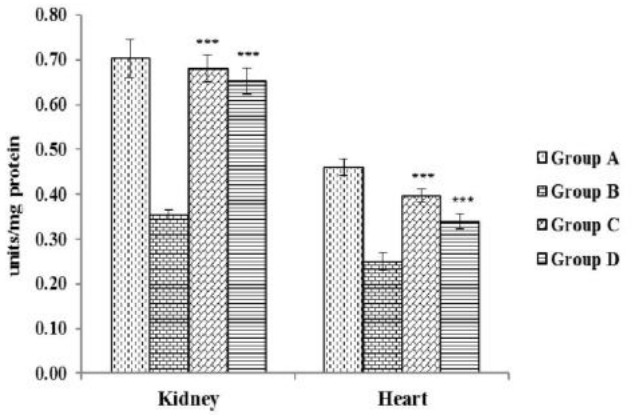

Figure 5. Effect of CHL and ascorbic acid on Mn-SOD activity in kidney and heart tissues of STZ-induced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: ***P < 0.001 as compared to the Group B.

The activity of CAT has been significantly increased when compared to the reference diabetic control group in both ascorbic acid-treated diabetic mice of Group C (0.91 ± 0.04 vs 0.64 ± 0.05 in kidney and 0.26 ± 0.01vs 0.10 ± 0.01in heart) and CHL-treated diabetic mice of Group D (0.88 ± 0.06vs 0.64 ± 0.05in kidney and 0.22 ± 0.02vs 0.10 ± 0.01in heart)as shown the result in figure6.

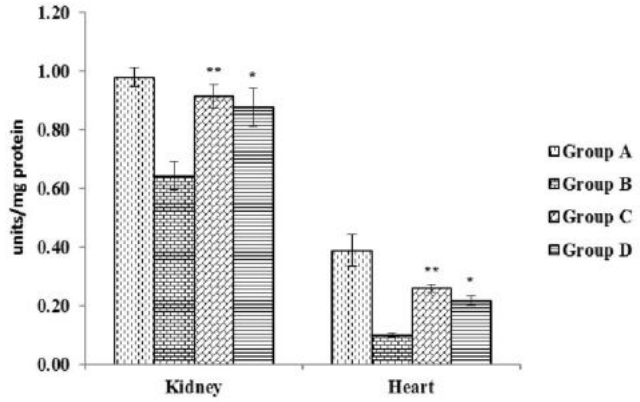

Figure 6. Effect of CHL and ascorbic acid on CAT activity in kidney and heart tissues of STZinduced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: *P < 0.05 and **P < 0.01 as compared to the Group B.

As shown the result in figure7, the activity of GPxwas found significantly higher in both ascorbic acid-treated diabetic mice of Group C (3.98 ± 0.5 vs 1.92 ± 0.3 in kidney and 1.80 ± 0.1 vs 0.96 ± 0.1 in heart) and CHL-treated diabetic mice of Group D (3.80 ± 0.3vs 1.92 ± 0.3in kidney and 1.70 ± 0.2vs 0.96 ± 0.1in heart)when compared to the diabetic mice of Group B in our present study.

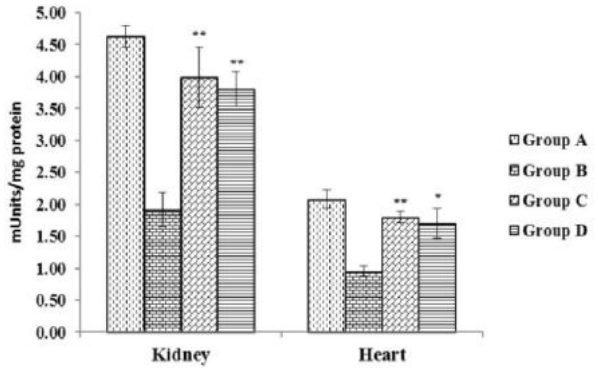

Figure 7. Effect of CHL and ascorbic acid on the GPx activity in kidney and heart tissues of STZ-induced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: *P < 0.05 and **P < 0.01 when compared to the Group B.

The activity of GR has been significantly increased when compared to the diabetic mice of Group B in ascorbic acid-treated diabetic miceof Group C(0.25 ± 0.03vs 0.17 ± 0.01 in kidneyand 0.09 ± 0.01vs 0.04 ± 0.01 in heart) and CHL-treated diabetic miceof Group D (0.27 ± 0.02vs 0.17 ± 0.01 in kidney and 0.08 ± 0.01 vs 0.04 ± 0.01 in heart)as shown the result in figure8.

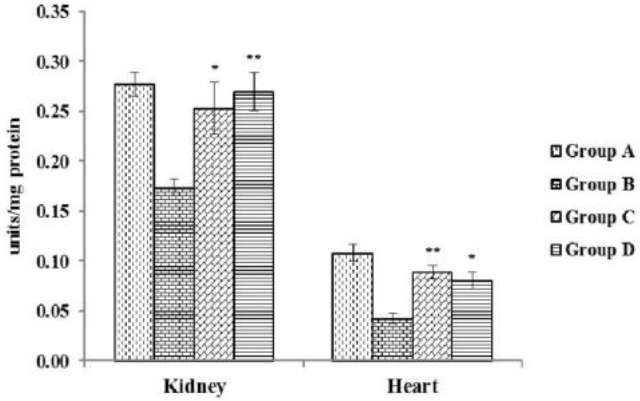

Figure 8. Effect of CHL and ascorbic acid on GR activity in kidney and heart tissues of STZinduced diabetic mice. Values are expressed as mean ± SEM; n = 6. Significant difference: *P < 0.05 and **P < 0.01 as compared to the Group B.

Histological study

As shownthe result in figure9,the glomerulus has been shrunken with the widening of bowmen's capsule space in thekidney of diabetic mice of Group B as compared to the reference normal control group. The kidney was less altered showing the normal physiological structure of glomerulus without any widening of bowman's capsule space in both ascorbic acid-treated diabetic mice of Group C and CHL-treated diabetic mice of Group D in our present study.

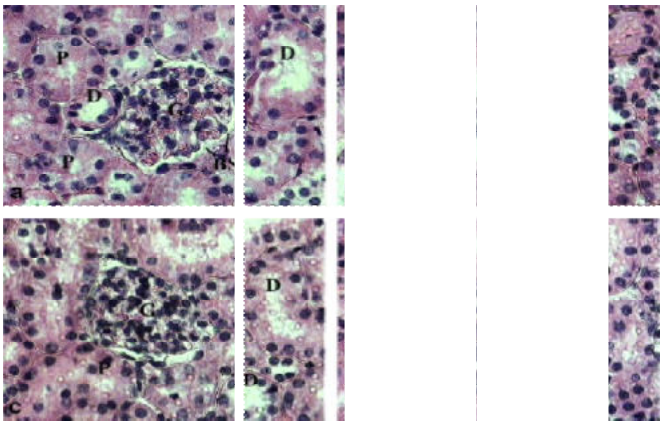

Figure 9. Photomicrographs of histological changes in kidney tissues. a: normal mice of Group A, b: diabetic mice of Group B, c: diabetic mice treated with the ascorbic acid of Group C, and d: diabetic mice treated with CHL of Group D. G: glomerulus, D: distal convoluted tubule, P: proximal convoluted tubule, BS: bowmen's capsule space, and arrow showing dilatation in BS.All images are under40x magnification.

As shown the result in figure 10, the heart tissue with enlarged interstitial space has been distorted in reference diabetic control group as compared to the normal mice of Group A. However, in the case of ascorbic acid-treated diabetic mice of Group C and CHL-treated diabetic mice of Group D, heart tissue was less altered showing no enlargement in the interstitial space.

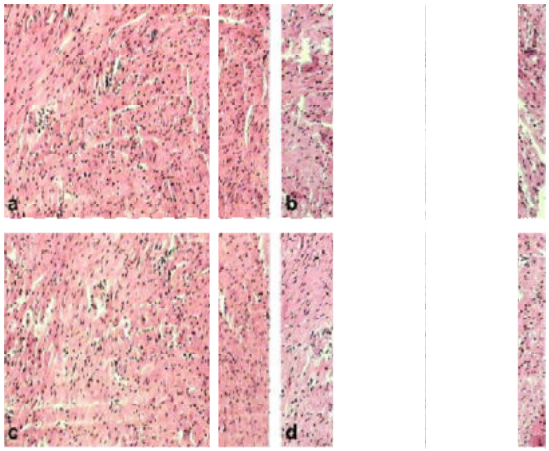

Figure 10. Photomicrographs of histological changes in heart tissues.a: normal mice of Group A, b: diabetic mice of Group B, c: diabetic mice treated with the ascorbic acid of Group C, and d: diabetic mice treated with CHL of Group D. All images are under10x magnification.

Ultrastructural study

As shown the result in figure 11,thickening of glomerular basement membrane and decreased number of podocytes were observed in renal corpuscles in the case of reference diabetic control group. The treatment of diabetic mice with ascorbic acid and CHL were able to restore the structure of glomerular basement membrane and podocytes number as observed in TEM images of renal corpuscles in both Group C and Group D respectively.

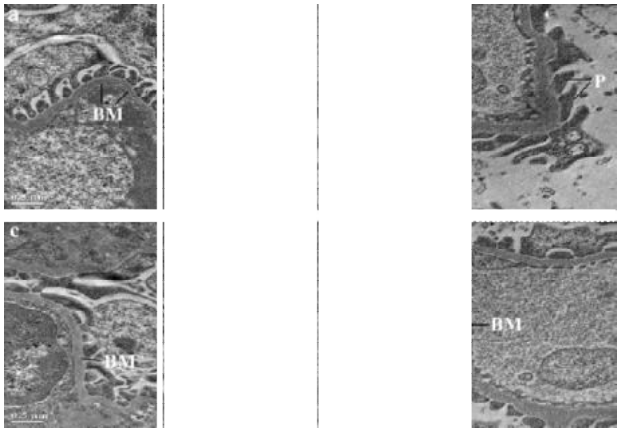

Figure 11. Electron micrographs of ultra structural changes in renal corpuscles. a: Normal mice of Group A, b: diabetic mice of Group B, c: diabetic mice treated with the ascorbic acid of Group C, and d: diabetic mice treated with CHL of Group D. BM: glomerular basement membrane and P: podocytes.

Mitochondria were seen ruptured and myofibrils were completely distorted in cardiomyocyteof reference diabetic control group, whereas cardiomyocyteswere less altered showingwelldistributed mitochondria and myofibrils with visible Z-line in thecase of the ascorbic acidtreated and CHL-treated diabetic mice ofGroup C and Group Das shown in figure 12 in our present study.

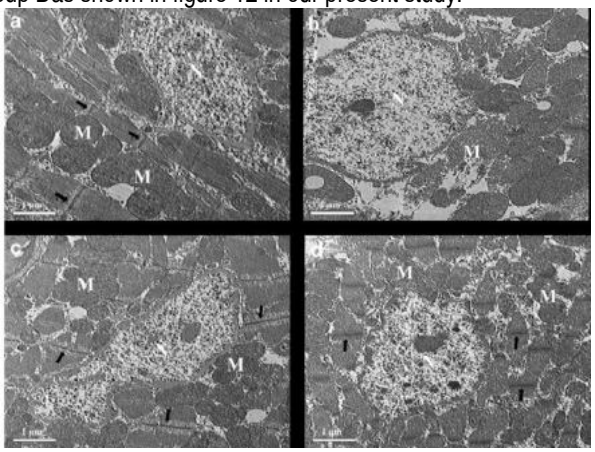

Figure 12. Electron micrographs of ultrastructural changes in cardiomyocyte.a: Normal mice of Group A, b: diabetic mice of Group B, c: diabetic mice treated with the ascorbic acid of Group C, and d: diabetic mice treated with CHL of Group D. N: nucleus, M: mitochondria, and black arrows showing Z-line

Discussion

It is well studied that biomolecules are more vulnerable to be attacked by ROS during oxidative stress. During lipid per oxidation, ROS impairs polyunsaturated fatty acids in theplasma membrane and elevates the concentration of TBARS, which is considered as an index for the study of oxidative stress [22].Several studies have also reported an increase in the level of protein carbonyls during oxidative stress [23,24].Carbonylation of protein has occurred by direct oxidation of the residues of lysine, arginine, proline and threonine residues from the protein chain, by interaction with lipid peroxidation products with aldehyde groups (such as 4-hydroxy2-nonenal, MDA, 2-propenal), or by the interaction with compounds with the carbonyl groups resulting from the degradation of the lipid or glycoxidation under oxidative stress condition [25].Our data are in consonance with reported literature that significant increase in ROS level with concomitant increased in MDA and protein carbonyl content implies oxidative stress in diabetic mice.It was observed that CHL was able to attenuate oxidative stress in diabetic mice by significantly reducing the level of ROS, MDA, and protein carbonyl in our study. In mammalian cells, superoxide anion($O_2^{\cdot-}$)that produced are first encountered and dismutate to $H_2O_2$by different isoforms of SOD, each is a product of the distinct gene and located in thedistinctintracellular environment. SOD generated H_2O_2 is neutralized by CAT in cytosol and peroxisome or by GPx in thecytosol subsequently.GR in cytoplasm does not act directly on ROS but assist GPxto function through converting GSSG to GSH and helps in maintaining a reduced intracellular environment. It was noted in our study that activity of CuZn SOD, Mn-SOD, CAT, GPx, and GR enzymes that takes part in antioxidant defense system, were significantly diminished in diabetic mice as compared to normal mice.This diminution of enzyme activity might be due to glycation of protein under the hyperglycemic condition of diabetes and/or lipid per oxidation which alter the trans-bilayer fluidity gradient that hampers the activities of membrane-bound enzymes and/or protein carbonylation which alter threedimensional structure of the protein and eventually activity. However, CHL increased the activity of CuZn SOD, Mn-SOD, CAT, GPx, and GR significantly in renal tissue of diabetic mice.The possible explanation behind this effect of CHL can be related to the scavenging property along with theability to prevent ROS-mediated chain reactions, inhibition of lipid peroxidation, and protein carbonylationprocess.

Another important finding of our study is that CHL has the ability to protect and restore tissues from detrimental effect of diabetes-induced oxidative stress, as evidenced by both histological and ultrastructural study. It was found in our study that tissues under thecondition of diabetesinducedoxidative stress exhibited etiological changes which contribute to theoccurrence of secondary complications in diabetes. However, it is also clearfrom the study that CHL is able to suppress oxidative stress preventing lipid per oxidation, protein carbonylation and enhancing the antioxidative enzymes activity, thus allowing tissues to recover from further impairments in diabetic mice.

Conclusion

In conclusion, the findings of the present study demonstrated that CHL treatment may provide beneficial effects against oxidative stress in STZ-induced diabetic mice.However, this study has not revealed the exact mechanism (s) of CHL's action and further investigation is underway to find out a clearer picture of the effects of CHL.

Acknowledgments

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Conflicts of interests

The authors declare no conflicts of interests.

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