5. Results:

In case of isocaloric diet of different dietary regime, no significant change was noticed in body weight in rats during the 90 days of experimental period. The average reno-somatic value (kidney weight/100g body wt) decreased significantly (Fig-1).

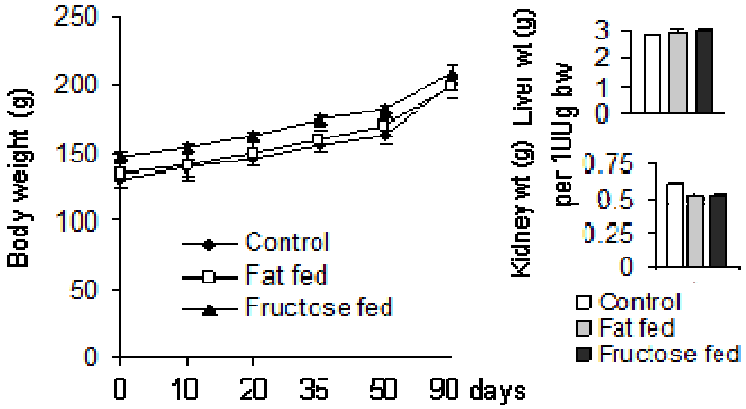

Fig-1. Follow-up of the body weight changes of rats maintained in different dietary regimen. Hepatosomatic and renosomatic index are shown in those rats.

The rat shows variation in their blood cholesterol and triglyceride level in both high lipid and fructose group with comparison to those of control groups (Fig-8). In tissue sample, both liver and heart tissue MDA level significantly increase in high lipid feed and fructose feed groups with respect to the corresponding control group but tissue NPSH level variations are less. The malondialdehyde (MDA) was increased in high lipid fed ($p<0.01$) or high fructose-fed ($p<0.001$) rat in the present study (Fig-8).

Our present data suggest that the catalase activity increased in liver tissues of high lipid-fed rats but this activity increased in heart tissue cytosol in either nutritional group (Fig-2). In

both tissues the standard oxidative stress generator menadione was efficient to activate either enzyme. The SOD1 activity appreciably increased in both tissues of rats from high lipid-fed group. But in fructose group the activity did not change and menadione slightly decreased the SOD1 activity in either tissue (Fig-2).

Fig-10 describes the serum MDA and NPSH levels in male and female cardiac patients with comparison to the normal individuals. MDA level was higher in case of cardiac samples than non-cardiac samples and NPSH level was also high in cardiac samples than non-cardiac sample. MDA and NPSH level was higher in case of female than male and vice versa was true for NPSH level. This result suggests that MDA increased appreciably both in male and female patients (40-50%) but the changes were not found significant due to higher intra-individual variability. The NPSH level did not alter in male patients and though not significant it increased ~50% in female patients.

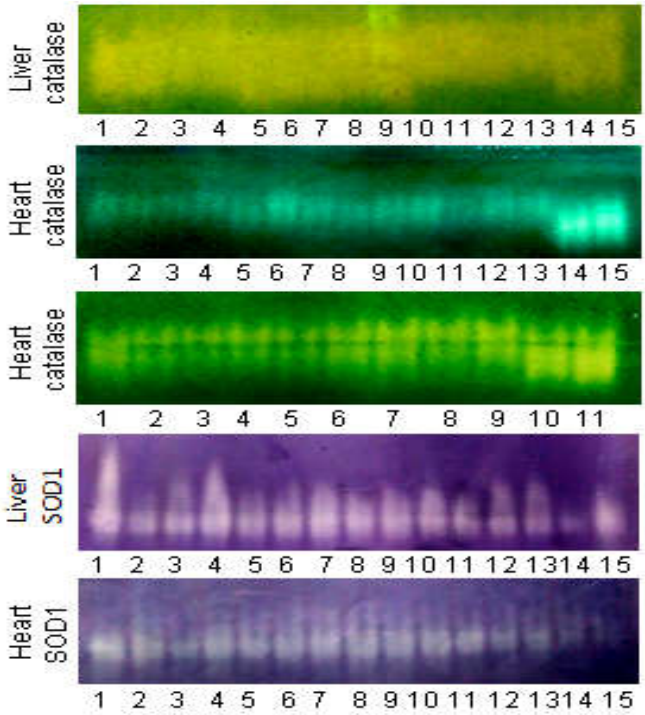

Fig-2. Lane distribution of catalase activity and superoxide dismutase activity (SOD1) of rat liver & heart tissues: liver catalase- lane 1-4 control, lane 5-9 high lipid feed, lane 10-13 high fructose feed, lane 14&15 menadione treated. Heart catalase- lane 1-4 control, lane 5-9 high lipid feed, lane 10-13 high fructose feed, lane 14&15 menadione treated. Another one gel heart catalase- lane 1-4 control, lane 5-9 high lipid feed, lane 10-11 menadione treated; superoxide dismutase activity: liver SOD1- lane 1-4 control, lane 5-9 high lipid feed, lane 10-13 high fructose feed, lane 14&15 menadione treated. Heart SOD1- lane 1-4 control, lane 5-9 high lipid feed, lane 10-13 high fructose feed, lane 14&15 menadione treated.

Serum sample was run in PAGE and the protein pattern is presented in a represented in picture (Fig-3). It demonstrates the trop T band in the lane 5 (female), 6 and 7 (male) which correspond to the 35 kDa marker shown in lane one. Fig-9 demonstrates the NBT reduction test on a polyacrylamide gel zymogram band made by serum SOD1 of trop T+ patients. It is evident from the picture that the SOD1 activity is very high (in lane 4 and 5, female and male respectively) in trop T+ patients serum sample. Some of the very high risk factors (high triglyceride and/or cholesterol or trop T-) patients' serum also generated mild SOD1 activities. SOD activity was higher in cardiac sample than non cardiac sample (Fig-9).

Present result suggests that serum SGOT and LDH in female cardiac patients significantly increased in both trop T- or trop T+ patients with comparison to the corresponding control values (table-1). The increase of SGOT in trop T+ vs. trop T- was found to be highly significant. But the increase of LDH (though it is ~45%) in same group was not found to be significant due to higher inter-individual variability. The CPK was found to be significantly higher ($p<0.001$) in trop T+ or negative groups vs control group ($p<0.001$). The CPK-MB was found to be significantly higher in trop T positive vs control group and in trop T positive vs negative group ($p<0.001$). Components of the lipid profile were found to be significantly increased ($p<0.05$ to $p<0.001$) in trop T+ or negative group vs control and trop T+ vs. – group except that in LDL of trop T+ vs control group (table-1). The multiple comparison ANOVA statistics data suggest that there is a significant difference in three groups (control, trop T- and trop T+) in case of most of the markers and risk factors except VLDL (table-1).

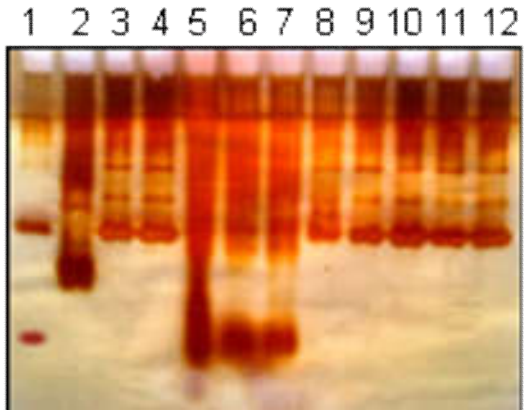

Fig 3. Representative protein band pattern in PAGE from serum sample of cardiac patients, control individuals and individuals with some risk factors. Silver staining was performed for the protein band visualization. Lane-1 denotes BSA, Lane-2 Egg Albumin, Lane-3&4 Control, Lane-5, 6&7 Trop-T positive, Lane-8&9 high Triglyceride, Lane-10 high cholesterol, Lane-11 high Triglyceride, Lane-12 high Sugar.

The results in the male cardiac patients suggest that SGOT, LDH, CPK-MB and HDL significantly ($p<0.001$) increased in trop T+ vs corresponding control groups and that value was found to be significant ($p<0.01$) in case cholesterol (table-2). Only the LDH and HDL were found to be significantly changed in trop T- group vs to those of control group. In some other cases like SGOT, CPK and CPK-MB very high inter-individual variability is noticed. A larger number of sample studies were generated some conclusive statistical outcome. Increases in these parameters in trop T+ vs control group were found to be appreciably higher (6 fold, 10 fold and 2.5 fold respectively). In case of trop T+ vs trop T- group, a significant increases ($p<0.05$ to $p<0.001$) were noticed in the parameters like LDH, CPK-MB, HDL and cholesterol (table-2). The multiple comparison ANOVA statistics data suggest that there are moderate and significant differences in three groups (control, trop T- and trop T+) in case of markers and risk factors except SGOT, CPK, LDL, VLDL and triglycerides (table-2). When the values of male and

female were compared no significant differences were observed between them except the control LDL level, which is in male found to be significantly ($p<0.01$) higher than that of female control.

Parameters

Groups	**sgot**	**ldh**	**cpk**	**cpk-mb**	**hdl**	**ldl**	**vldl**	**cho**	**tri**
Control (n=9)	43.88	112.23	56.58	17.63	49.15	69.62	22.68	117.86	77.38
	±5.67	±9.89	±5.39	±2.34	±3.46	±4.52	±3.31	±8.44	±6.35
Trop T-	89.21[a]	437.36[b]	402.10[c]	35.47	37.02	68.19	59.86[a]	169.92[b]	118.26[c]
(n=12)	±20.97	±102.23	±129.31	±8.55	±5.01	±7.66	±18.31	±15.60	±8.53
Trop T+	361.06[cm]	631.47[c]	435.68[c]	118.45[cm]	122.05[cm]	89.55[cm]	44.20[bl]	247.15[cl]	148.45[cl]
(n=11)	±59.34	±65.37	±77.12	±13.94	±19.59	±4.77	±6.46	±21.57	±12.31
ANOVA (F) Stat	**22.18**	**11.49**	**4.717**	**30.86**	**16.07**	**4.232**	**2.27**	**15.39**	**13.67**
(P)	**0.001**	**0.001**	**0.017**	**0.001**	**0.001**	**0.024**	**0.122**	**0.001**	**0.001**

Table-1. Several hematopoietic biochemical parameters like risk factors and markers of cardiac disease were measured by standard kit method in female cardiac patients. Sgot- serum glutamate oxaloacetate transaminase, ldh- lactate dehydrogenase, cpk- creatinine phosphokinase, cpk-mb- creatinine phosphokinase-mb, hdl-high density lipoprotein, ldl-low density lipoprotein, vldl- very low density lipoprotein, cho- cholesterol, tri- triglyceride. Sample sizes are mentioned in the 'group' column. The values are denoted as mean ± SE. Group-wise comparison (trop T- or trop T+ vs control) by students't test and the level of significance are presented beside the mean as a superscript. a = $p<0.05$, b = $p<0.01$, c = $p<0.001$. When comparison is made between trop T- and trop T+ vs, the level of significances are k = $p<0.05$, l = $p<0.01$, m = $p<0.001$.

Parameters

Groups	**sgot**	**ldh**	**cpk**	**cpk-mb**	**hdl**	**ldl**	**vldl**	**cho**	**tri**
Control	49.26	132.76	68.27	21.36	45.67	98.84	26.34	138.52	86.79
(n=12)	±2.48	±7.98	±3.43	±3.22	±2.44	±6.77	±2.35	±10.97	±4.56
Trop T-	307.40	347.20^{c}	685.08	52.08	36.75^{a}	99.25	23.00	159.38	109.13
(n=23)	±201.69	±43.41	±443.12	±22.60	±4.32	±16.61	±5.10	±17.52	±26.66
Trop T+	253.38^{c}	735.62^{ck}	430.36^{c}	172.26^{cm}	181.17^{cm}	90.88	38.64	312.35^{bl}	125.22
(n=14)	±42.25	±101.50	±69.75	±26.00	±40.01	±9.45	±6.45	±39.12	±18.73
ANOVA(F)	**0.62**	**21.58**	**0.72**	**11.31**	**16.45**	**0.10**	**2.48**	**14.17**	**0.543**
Stat (P)	**0.543**	**0.001**	**0.49**	**0.001**	**0.001**	**0.904**	**0.095**	**0.001**	**0.585**

Table- 2. Several hematopoietic biochemical parameters like risk factors and markers of cardiac disease were measured by standard kit method in male cardiac patients. Sgot- serum glutamate oxaloacetate transaminase, ldh- lactate dehydrogenase, cpk- creatinine phosphokinase, cpk-mb- creatinine phosphokinase-mb, hdl-high density lipoprotein, ldl- low density lipoprotein, vldl- very low density lipoprotein, cho- cholesterol, tri-triglyceride. Sample sizes are mentioned in the 'group' column. The values are denoted as mean ± SE. Group-wise comparison (trop T- or trop T+ vs control) by students't test and the level of significance are presented beside the mean as a superscript. a = $p<0.05$, b = $p<0.01$, c = $p<0.001$. When comparison is made between trop T- and trop T+ vs, the level of significances are k = $p<0.05$, l = $p<0.01$, m = $p<0.001$.

The distribution and dispersion pattern of nutritional status represented by BMI of both sexes among the three groups of participants, classified as their blood glucose level (viz. ≤140, 141–270, ≥270 mg/dL) are shown in Fig-4a. Nutritional status (BMI) and age wise distributions of male and female blood glucose data are shown in a typical scattered plot Fig-4b. When the studied group are divided into two age groups (≤40 and ≥40 years). It was found that the blood glucose is higher of the participants of ≤40 years than ≥40 years (mean ±SE 258.75±10.18 and 212.48±10.67 respectively, p=0.004) Fig-12b.

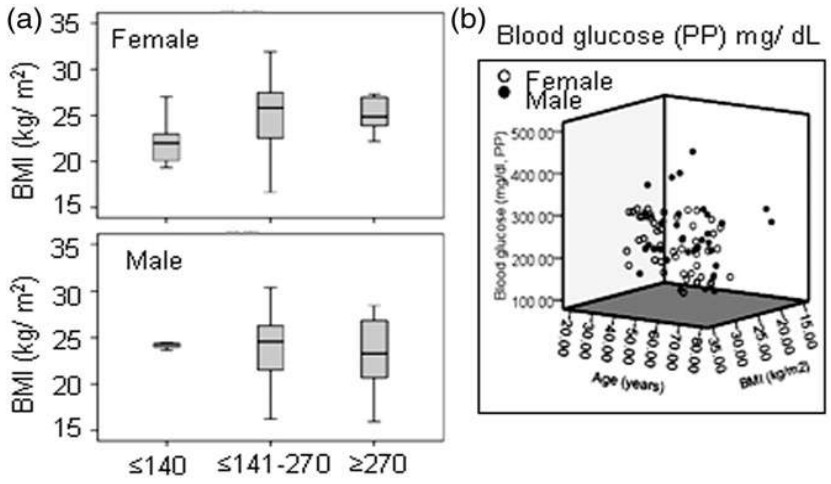

Fig-4. Nutritional status (BMI) wise distribution and dispersion pattern in female and male data from different groups of blood glucose level.

Present results indicate that the mean±SE glucose levels (PP) are 305.50±21.35, 217.58±1.34 and 226.94±9.59 mg/dL in underweight, normal weight or overweight categories respectively (F=6.357, p<0.003) Fig-4a. While participants are grouped based on their diabetic severity (≤140, 141–270, ≥270 mg/dL PP), it is noticed that unlike normal or overweight groups, all malnourished individuals occupy 141–270 and ≥270 mg/dL glucose groups Fig-11. The distributions of participants in overweight and underweight groups are 38.89% and 11.11%. A

trend of higher blood glucose level (>270 mm/dL) is seen among underweight participants (60%), while among the normal and overweight participant it is 26.67% and 31.43% respectively Fig-4.

5.1. Effect of DCN-2 in the synthesis of TNF-α and IL-6 in normal neutrophils: Incubation of different concentrations of DCN-2 to the neutrophils have shown to elevate TNF-α and IL-6 level in the solution. It has been found that TNF-α level has been increased from 3.829 ± 1.53 pg/ml to 20.7 ± 6.9 pg/ml due to incubation of 130 nM DCN-2 to the neutrophils solution for 120 min at 37°C Fig-5a. The incubation of 0.6 nM estriol before the treatment with different concentrations of DCN-2 resulted to produce a decreased level of TNF-α with 11.97 ± 0.92 pg/ml due to incubation in the same environment Fig-5a.

Expression of TNF-α was also determined by Western blot technique and the bands

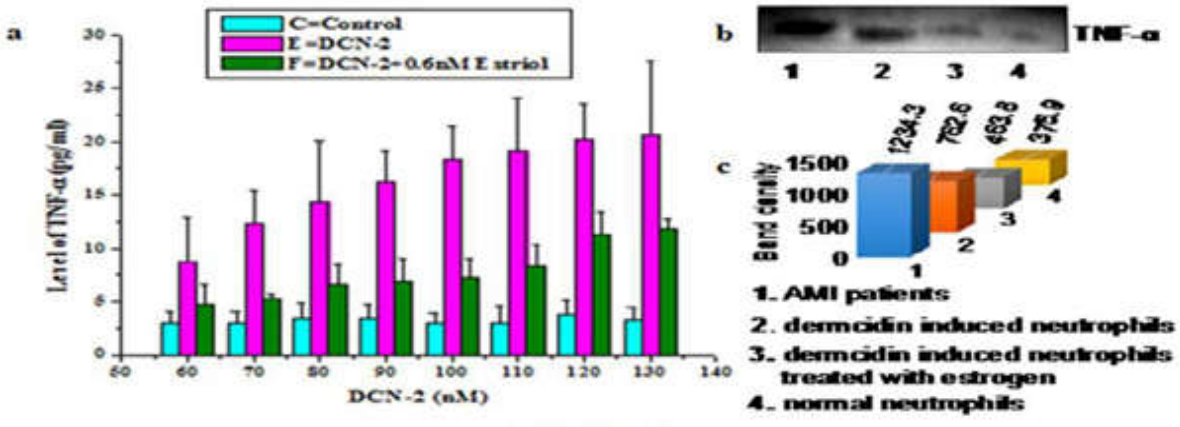

Fig-5 (a) DCN-2 induced synthesis of TNF-α. Incubation of DCN-2 to the neutrophils was followed by the determination of TNF-α. Control group (C) shows the synthesis of TNF-α in the absence of DCN-2. Group E describes the synthesis of TNF-α in the presence of different concentration of DCN-2. In the next group (Group F) the neutrophils were pre-incubated with 0.6 nM estriol and different concentrations of DCN-2 then. (b) Expression of DCN-2 induced TNF-α in neutrophil represented by western blot technique. Lane 1 describes TNFα expression in neutrophil from AMI patients. Lane 2 shows TNFα level in dermcidin induced normal neutrophils. Lane 3 describes TNFα level in dermcidin induced normal neutrophils treated with estrogen. Lane 4 describes TNFα level in normal neutrophils. (c) Measurement of the intensity of band from western blotted film. Band intensity was measured by software ImageJ. Lane 1 shows the intensity of TNF-α protein (blood collected from AIHD patients having DCN-2 level 142.49 ± 17.38 nM) 1234.44 Units. Lane 2 describes the band intensity of the normal neutrophil (primary DCN-2 level 38.44 ± 8.34 nMin age and sex matched sample of the AIHD patients). It increased to 762.72 Units from 375.74 Units (Lane 4) due to treatment of added 120 nM of DCN-2 to the neutrophil solution. Lane 3 demonstrates a decreased level of TNF-α expression (463.28 Units) from 762.72 Units in Lane 2 as 0.6 nM estriol was incubated to DCN-2 treated (120 nM added) normal neutrophil

obtained on the photographic films have described different level of intensity Fig-5b. It was found that the band intensity for the TNF-α protein in AIHD patients was highest as 1234.44 Units Fig-5c. Whereas in neutrophil obtained from age and sex matched normal control was found to be 375.74 Units which increases to 762.72 Units as 120 nM of DCN-2 was incubated to normal neutrophil solution for 2 h at 37°C Fig-5c. A 45 min treatment of 0.6 nM estriol after 120 nM DCN-2 treated neutrophil solution the expression of TNF-α was inhibited and the band intensity was 463.28 Units Fig-5c.

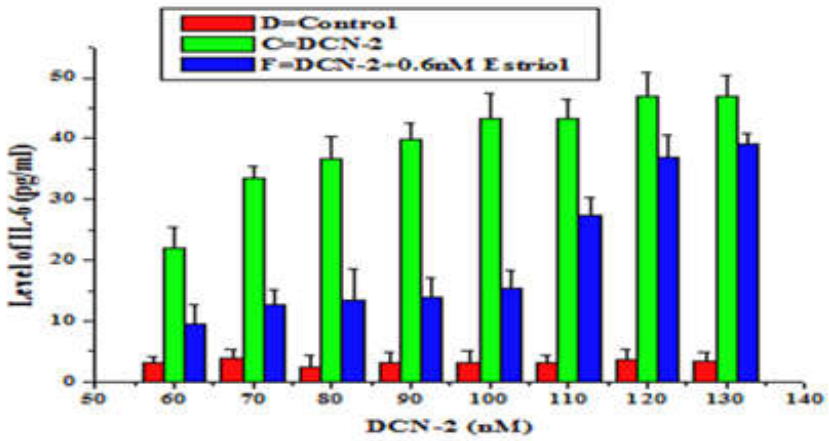

Fig-6: Synthesis of IL-6 in neutrophils by DCN-2. The level of cytokine IL-6 was determined by ELISA. Here, Group D denotes the synthesis of IL-6 in control. Group-C describes the synthesis of IL-6 in 120 min incubated neutrophils. In group F, the synthesis of IL-6 was characterised after incubating 0.6 nM estriol followed by different concentrations of DCN-2.

The level of IL-6 has been changed from 3.27 ± 1.52 pg/ml to 47.07 ± 3.4 pg/ml due to incubation of 130 NM of DCN-2 to the neutrophils for 120 min at 37°C Fig-6. The inhibitory effect of 0.6 nM of estriol for 45 min pre-incubation has shown a decreased level of IL-6 to 15.34 ± 2.91 pg/ ml from 43.218 ± 4.2 pg/ml Fig-6. But gradual increase of DCN-2 to the

neutrophil solution was found to nullify the inhibitory effect of 0.6 nM estriol on IL-6 level. And, at the concentration of 130 nM DCN- 2 the cytokine IL-6 level was 47.07 ± 3.4 pg/ml whereas 0.6 nM estriol was able to restore the level to 39.03 ± 1.82 pg/ml

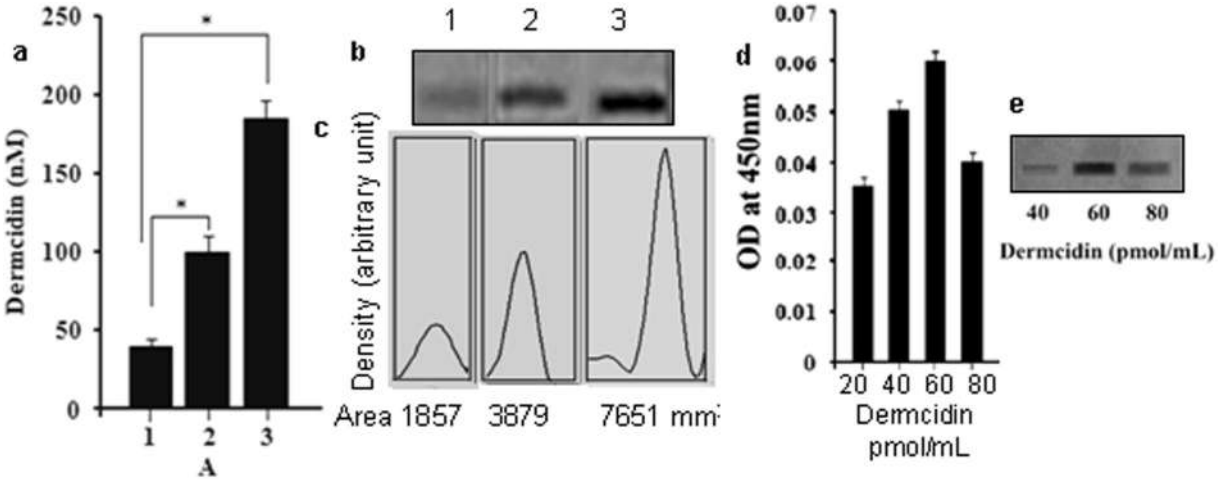

Fig-7. Identification of dermcidin in the cell free plasma of both normal and type-1 diabetes mellitus patients. The cell free plasma from both normal and diabetic patients was prepared as described in the Materials and Methods section. The amount of dermcidin was determined by ELISA by using polyclonal antibody raised against the purified dermcidin. Panel-a: Solid bar '1' represents the dermcidin level in normal subject. Solid bar '2' and '3' represents the dermcidin level in T1DM patients with blood glucose level in between 120–340 mg/ dL and > 340 mg/dL respectively. Panel-b: Immunoblot analysis of the plasma level of dermcidin in normal and T1DM subjects. '1' represents the dermcidin band in the cell free plasma (CFP) of normal subject. '2' and '3' represents the dermcidin bands in the CFP of T1DM subjects with blood glucose level in between 120–340 mg/ dL and >340 mg/dL respectively. Data are mean ± S.D. of at least 10 different experiments using 10 different samples from 10 different volunteers (Male = 5, Female = 5) (*represents $p < 0.05$). Panel-c: Integrated area of each of the immunopositive band as shown in the Panel-b. Panel-d: A standard bar diagram of ELISA assay for pure dermcidin protein. Dermcidin was purified by repeated gel electrophoresis as described. The ELISA assay of the purified dermcidin was conducted by using commercial dermcidin antibody. Panel-e: Western blot analysis of purified dermcidin by using commercial dermcidin antibody.

5.2. Plasma level of dermcidin in type I diabetes mellitus (T1DM) subjects:

It was found that the patients with blood sugar level > 340 mg/dl had higher level of the stress induced protein (180 pmol/mL). Those T1DM patients who have blood sugar level in between 120–340 mg/dL the plasma DCN-2 level was 100 pmol/ mL. But in the normal controls

it was found that the plasma DCN-2 level was much less as compared to the T1DM subject (40 pmol/mL) Fig-7a. It was found that the expression of DCN-2 immunopositive band was very high in the T1DM patients (Fig. 7c, lane-2 and 3). But in case of normal controls the expression of DCN-2 was less compared to the T1DM subject Fig-7b, lane-1.

5.3. Plasma level of C-reactive protein (CRP) in both T1DM and normal subject:

It has been reported that the insulin sensitivity is negatively correlated with circulating high sensitive CRP (hs-CRP) (Apostolopoulou et al., 2016), and chronic inflammation due to activation of hs-CRP may play a role in changes in glucose homeostasis in T2DM (Deichgræber et al., 2016). It was found that patients with blood glucose level > 340 mg/dL had higher level of CRP in the plasma 90 µg/mL. Those patients who had blood sugar level in between 120–340 mg/dL, the plasma CRP level were 80 µg/mL. But in normal controls it was found that the plasma CRP level was much less 20 µg/mL compared to the T1DM subject Fig-15A.

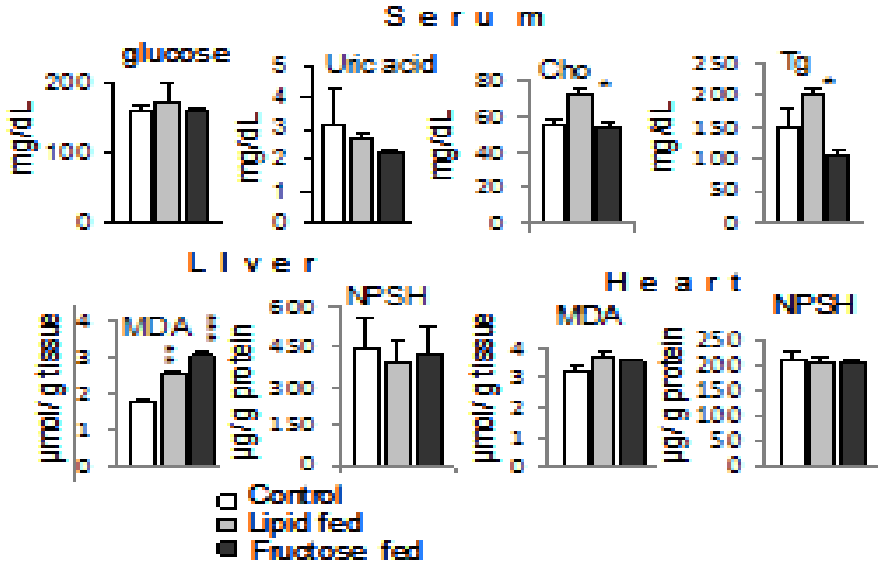

Fig-8. Bar diagram shows comparison of blood glucose, uric acid, cholesterol and triglyceride of male Wister rats, feeding isocaloric diet: high lipid feed diet and fructose diet with the corresponding control group feeding isocaloric diet. MDA and NPSH was compared with both liver and heart tissue sample among the atherogenic, fructose feed and control group. The values are denoted as mean ± SE.

5.4. Plasma level of malondialdehyde (MDA) in both T1DM and normal subject:

It was previously reported that inflammatory responses such as IL-6 and TNF-α accumulated oxidative damage products in the liver of diabetes mellitus (DM) patients (Mohamed et al., 2016). It was found that plasma MDA level was 0.2 μmole/100 μL in the T1DM subject with blood glucose level > 340 mg/dL, which was very high than other groups Fig-15B.

5.5. Plasma level of non-protein soluble thiol (NPSH) in both normal and T1DM subject:

NPSH improve insulin resistance and exhibit significant reductions in serum free fatty acids, oxidative stress and inflammatory parameters in diabetic patients (Salman et al,. 2013). It was observed that normal patients had higher level of NPSH 0.85 μg/100 μL in their plasma where as diabetic patients have very low amount of NPSH 0.4 μg/100 μL in their plasma compared to the normal control Fig-15C.

5.6. Effect of dermcidin isoform-2 in the synthesis of TNF-α in liver cell: Incubation of different concentrations of DCN-2 to the liver cell has shown to elevate TNF-α in the incubation mixture. It was found that the TNF-α level was increased from 2.59 ± 1.53 pg/mL to 6.8 ± 1.6 pg/mL due to incubation of 0.2 μM dermcidin to the liver cell homogenate for 60 min at 37 °C Fig-15D. The control had a very low TNF-α.

5.7. Role of estriol to reduce the level of TNF-α and IL-6 in neutrophils from AIHD:

Neutrophils from AIHD (n=9, M=7, F=2) patients were collected and was assayed for both TNF-α and IL-6 level. It is noticed that TNF-α level was 18.3-27.3 pg/ml, median value 21.863 pg/ml Fig-13 and IL-6 was 23.54-52.733 pg/ml, median value 42.163 pg/ml (Figure 14) in CAD patients. Age and sex matched controls have 0.29-1.54 pg/ ml, median value 0.98 pg/ml

TNF-α level Fig-13 and IL-6 level was 1.56-5.923 pg/ml, median value 4.433 pg/ml Fig-14. Incubation with 0.6 nM estriol for 45 min at 37°C showed level of both cytokines TNF-α andIL-6. A high level of TNF-α and IL-6 in CAD patient have been found to be declined to 4.9-9.13 pg/ml, median value 7.43 pg/ml Fig-13 and 7.91-21.873 pg/ml, median value 16.733 pg/ ml Fig-14 respectively for TNF-α and IL-6 after treating with estriol as described aboveGroup study of the blood serum of cardiac risk factors and cardiac marker with control the level of hs-CRP(inflammatory marker) and T3(metabolic hormone) both are significantly increase in high cholesterol and Trop T+ groups with respect to the corresponding control group Fig-16&17 but T4 and TSH level variations are less.

5.8. Correlation between plasma dermcidin level with plasma NO and insulin levels in hyperglycemic and normoglycemic subjects:

It was noticed that T1DM subjects had lower levels of plasma NO and insulin when compared with the age and sex matched normal controls Fig-18A, B. The plasma NO and insulin levels in T1DM were highly and negatively correlated Fig-18 C, D and E, (coefficient of correlation 'r' = −0.9899). From Fig-18D, it was found that plasma NO levels in diabetic patients (n = 30) were 0.5 nmol/mL (median ranging from 0.1 nmol/mL to 1.3 nmol/mL) and the plasma NO level in normal subjects were 4 nmol/mL (median ranging from 2.5 nmol/mL to 43 nmol/mL). Plasma insulin level in T1DM patients was 1.5 μU of insulin/mL (median ranging from 0.02 μU of insulin/mL to 2.2 μU of insulin/mL) and plasma insulin level in normal subject was 14 μU of insulin/mL (median ranging from 7 μU of insulin/mL to 13.7 μU of insulin/mL) Fig-18E.

5.9. Effect of DCN-2 in the inhibition of estriol induced NO production in normal neutrophils:

Different concentrations of DCN-2 were incubated to the neutrophils for 120 min at 37°C to find out if the DCN-2 has any inhibitory property to estriol induced NO production. Neutrophils which were pre-treated with 0.6 nM estriol for 45 min at 37°C also subjected for NO assay with different concentrations of DCN-2. It was found that incubation of 120 nM DCN-2 to the neutrophils caused a distinctive inhibition of the NO production from 1.61 nmol NO/ ml to 0 nmol NO/ml. NO level was also decreased to 0.559 nmol/ ml in post treatment of 0.6 nM estriol after incubating with different concentration of DCN-2 Fig-19. But the pre-treatment of 0.6 nM estriol showed the NO level 1.27 nmol/ml which did not affect too much due to incubation with DCN-2 Fig-19.