

Apoptosis of Cardiomyocytes Assessed by a Novel Apoptotic Marker (Annexin V) and the Role of Vitamin E in Cardioprotection in Diabetic Rats

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Abstract

Background: Recently it has been shown that annexin V is an apoptotic marker in the heart. The aim of the present study was to explore whether annexin V content in the heart changes during diabetes in order to use it as a prognostic factor in diabetic cardiac affection. In addition, the study attempted to show the effect of treatment of insulin and vitamin E (300mg and 600mg/Kg) used separately or in combination.

Methods: The study included 70 male albino rats weighing 170-200gm. They were divided into the following groups of 10 rats each: Group I (cont): Control group, Group II (DM): Diabetic, untreated group. Diabetes was induced by intraperitoneal injection of streptozotocin (stz) at a dose of 40mg/kg body weight, Group III (Ins): Diabetic group, treated with 1 unit insulin injected subcutaneously, Group IV (E-300): Diabetic group, receiving 300mg/kg vitamin E injected intramuscularly, 3 times/week for one month, Group V (E-600): Diabetic group, receiving vitamin E i.m injection at a dose of 600mg/kg, 3 times/week for one month, Group VI (I+E-300): Diabetic group treated with insulin and vitamin E (300mg/kg/dose) and Group VII (I+E-600): Diabetic group treated with insulin and vitamin E (600mg/kg/dose). Annexin V and inducible nitric oxide synthase (iNOS) were examined in the hearts of animals by RT-PCR. Also, cGMP, malondialdehyde (MDA), glutathione peroxidase (GPX) and cardiac enzymes were measured.

Results: There were three major new findings in the present study. First; annexin V levels were significantly elevated in the heart of diabetic rats compared to controls. This elevation in annexin V content indicates that apoptosis is the basis of cardiac injury as shown by the elevated cardiac enzymes AST, ALT & CPK. Second, levels of iNOS protein of the heart were elevated in diabetic rats. On the basis of these findings, we can suggest that inflammation could play a role in the pathogenesis of diabetic cardiomyopathy in type 1 diabetes. Third, the effect of various types of treatment of diabetes which were used here showed the beneficial effects of the use of the antioxidant, vitamin E.

Conclusion: These results suggest that annexin V can be used as a prognostic factor in cardiac affection in DM. In addition, they provide further evidence that pharmacological intervention by different antioxidants may have significant implications in the prevention of the pro-oxidant features of diabetes.

Key Words: Annexin V – Diabetes – Diabetic cardiomyopathy – Insulin – Oxidative stress – Vitamin E.

Introduction

ANNEXINS are defined as soluble hydrophilic proteins that bind to negatively charged phospholipids in a Ca²⁺ dependent manner. This binding is reversible and removal of Ca²⁺ by Ca²⁺ chelating agents will lead to liberation of annexin from phospholipid matrix [1].

Depending on Ca²⁺ concentration, it has been reported that Ca²⁺ participates in a variety of a membrane related events such as exocytosis, endocytosis, apoptosis and binding to cytoskeletal proteins. It has been also reported to regulate protein activities [2] and is also involved in Ca²⁺ signaling. Recently it was shown that annexin V is the most prominent member of the annexin family in the adult heart. It was found that annexin V protein levels declined during maturation. Moreover, the subcellular localization of annexin V might change from cytoplasm to more prominent sarcolemmal localization during cardiac maturation [3].

Annexin V is mainly localized in cardiomyocytes. However, it could be relocated to interstitial tissue in ischemic and failing hearts or it could be externalized and exhibit a proapoptotic effect in cardiomyocytes [2].

It was found that early apoptosis can be assessed and imaged with annexin V scintigraphy in rats [4,5],

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based on its ability to identify extracellular phosphatidyl-serin, which arises during apoptosis [6].

It is found that there is significant increase of plasma annexin V concentration in patients with acute myocardial infarction which could reflect the severity of damage of myocardium [7].

It is known that insulin-dependent (Type 1) diabetes is a prevalent disease that increases the incidence and severity of cardiovascular complications [8].

Oxidative stress, on the other hand, has been implicated in the pathogenesis of diabetes mellitus [9] due to increased oxygen free radical production. Oxidative stress, in part, results from hyperglycemia, but it may also precede and accelerate the development of diabetes and then of diabetic complications [10].

The aim of the present work was to assess whether apoptosis plays a relevant role in the development of diabetic cardiomyopathy using this novel apoptotic marker; annexin V.

Annexin V was measured by RT-PCR in the heart of diabetic rats.

In addition, we also aimed to show the role of vitamin E in cardioprotection on the basis that diabetes is accompanied by oxidative stress and impaired bioactivity of nitric oxide (NO) both of which play an important role in the pathogenesis of macro as well as microangiopathic complications in diabetes mellitus.

Material and Methods

A- Drugs:

- I- Streptozotocin (STZ), (Trade name Zanosar), purchased from Sigma chemical company, St. Louis, Missouri, USA, in the form of vials of 1gm. The drug was dissolved in 0.1M sodium citrate (pH adjusted to 4.5).
- II- Insulin (Actrapid HM) was purchased from Novo Nordisk chemical company, in the form of ampoules of 100IU/ml.
- III- Vitamin E was purchased from Pharco Pharmaceuticals company, in the form of ampoules of 250mg.

B- Experimental animals:

The present work was performed on 70 male albino rats, weighing 170-200gm. They were obtained from central animal care services at the Faculty of Medicine, Cairo University. Animals were housed on woodchip bedding in polycarbonate

cages and offered free access to both food and water. Animals were divided into 7 equal groups, each of 10 rats.

- Group I: Control group. These received a single intraperitoneal injection of phosphate buffered saline (PBS).

Diabetes was induced in all the experimental animals by a single intraperitoneal injection of STZ (40mg/gm body weight), freshly dissolved in 0.1 M sodium citrate (pH adjusted to 4.5) [11].

Blood samples were obtained from the retro-orbital vein for measurement of blood glucose 48 hours after injection of STZ. An animal with blood glucose >250mg/dl was considered to be diabetic [11]. Blood was left for 30 minutes then centrifuged at 4000 rpm for 15 minutes at room temperature. Serum was collected and kept at -80°C till time of assay. Blood glucose was measured using commercial kit [11].

The diabetic groups were classified as follows:

- Group II (DM): Diabetic, untreated group.
- Group III (INS): Diabetic group, treated with 1 unit insulin injected subcutaneously, once daily, started 4-5 days after induction of diabetes.
- Group IV (E300): Diabetic group, receiving 300mg/kg Vit. E only, injected intramuscularly, 3 times/week for one month [12] started 4 days after induction.
- Group V (E600): Diabetic group, receiving vitamin E injection i.m at a dose of 600mg/kg, 3 times/week for one month [12] started 4-5 days after induction of diabetes.
- Group VI (I+E300): Diabetic group treated with insulin (1 unit sc daily) and vitamin E (300mg/kg, 3 times/week for one month), started 4-5 days after induction of diabetes.
- Group VII (I+E600): Diabetic group treated with insulin (1 unit sc daily) and vitamin E (600mg/kg, 3 times/week for one month), started 4-5 days after induction of diabetes.

All the animals were sacrificed by decapitation after 4 weeks of treatment. The hearts were obtained from all the animals.

C- Tissue preparation:

About 30mg of heart tissue was homogenized in RNA lysis buffer which contained mercaptoethanol after centrifugation at 10,000 rpm for 10min. The supernatant was kept frozen at -80°C till examined for gene expression of annexin V and

iNOS by RT-PCR. Another 20mg of heart tissue was homogenized in lysis buffer contain protease inhibitor, then centrifuge at 4.000 rpm for 5min. The supernatant was kept frozen till examined for MDA and glutathione peroxidase.

1- Gene expression of annexin and iNOS:

RNA extraction:

Total RNA was extracted from heart tissue homogenate using SV total RNA isolation system kit (Promega, Madison, WI, USA) according to manufacturer recommendations. Quality and quantity of RNA were determined by measurement of absorbance at 260nm using spectrophotometer.

Reverse transcriptase and polymerase chain reaction (RT-PCR):

cDNA was prepared from RNA as follows: About 20 μ g of mRNA were heated at 70°C for 5min with 50pmol of reverse primer of selected gene (annexin, iNOS) before adding 5XRT buffer (50mM Tris CL, pH 8.3, 10mM dNTPS and 200 units of murine leukemia virus reverse transcriptase in a final volume up to 36 μ l). RT reaction was carried out for 2 hours at 37°C.

Polymerase chain reaction (PCR):

5 μ l of cDNA were subjected to PCR under the conditions specified in Table (1); PCR reaction was carried out by adding 50pmol of each of forward and reverse primers specific to each gene as shown in Table (1).

10mM dNTPS, 2-5 units TACL polymerase, PCR 10 x buffer (containing 1 00mM Tris HCL pH 8.3, KCL 10mM to a final volume of 50 μ l).

Table (1)

Primer sequence	Cycling condition
1- <i>Annexin</i> :	94°C → 1min
Sense: 5' GTC TCC ACC CAC TTA	60°C → 1min
GTC TAA GTT-3'	72°C → 1min
Anti sense:	Extension
5' CCC TGA CAA TGA ACG CTG	72°C → 10min
CCA-3'	
2- <i>iNOS</i> :	95°C → 30sec
Sense 5' GTG AGG ATG AAA ACA	57°C → 45sec
TGG-3'	72°C → 45sec
Anti sense:	Extension
5' ACC TGC AGG TTG GAC CA-3'	72°C → 8min

Agarose gel electrophoresis:

The amplified PCR products of selected genes were electrophoresed on 1.5% agarose gel and were UV visualized after staining with ethidium bromide.

UV illuminated gel was photographed and densitometry using a standard DNA of known concentration, gene genius gel documentation system was used for analysis (Syngene, Cambridge UK). See Figs. (4,5).

2- Measurement of MDA:

MDA was measured in tissue homogenate after precipitation of protein by addition of trichloroacetic acid (TCA). Next, thiobarbituric acid (TBA) reacted with MDA to form thiobarbituric reactive product which was measured at 530nm according to [13].

3- cGMP was assayed using an ELISA kit (R & D USA) according to manufacturer recommendations [14].

4- Glutathione peroxidase (GPX) was assessed using Wak-Cheme cat. No: Wak-FR-GPX 80, Germany according to manufacturer recommendations.

5- Cardiac enzymes were measured using commercial kits.

Statistical analysis:

Results are presented as mean values \pm SD. The effects of diabetes and animal treatment were analyzed by one way ANOVA. When a significant F was obtained, multiple comparison post tests were used to determine which groups were significantly different. Probability values (p) <0.05 were considered significant. p values less than 0.01 were indicated separately.

Results

The results of the present study are summarized in Tables (2-5) and Figs. (1,2,3).

Table (2) shows that annexin levels were significantly elevated (p <0.001) in group II compared with controls levels were decreased significantly (p <0.001) in group III compared with group II, but were still higher than in controls. Also, in group IV mean annexin levels were lower than those in group II (p <0.01) but still higher than in controls (p <0.01).

In addition, the groups treated with both insulin and Vitamin E (groups VI and VII) showed significantly lower levels than those of group IV (p <0.01).

On the other hand, in groups VI and VII, mean levels of annexin V showed no significant differences when compared with each other and with controls but they showed significantly lower levels when compared with the untreated diabetic group II (p <0.01).

Table (2): Mean annexin V levels ($\mu\text{g}/\text{mg ptn}$) in the heart tissues of studied groups by RT-PCR (no=70).

Groups	Group I (cont)	Group II (DM)	Group III (INS)	Group IV (E300)	Group V (E600)	Group VI (I+E300)	Group VII (I+E600)
No.	10	10	10	10	10	10	10
Annexin	402.95± 97.295#	1042.85± 138.45*	564.2± 108.22##	656.96± 138.06##*	545.32± 105.58#	423.88± 65.76•#	424.87± 66.89•#

* Significant with Group I.

Significant with Group II.

• Significant with Group IV.

Group I (cont) : Controls.

Group IV (E300) : Diabetics, receiving 300mg/kg Vit. E only.

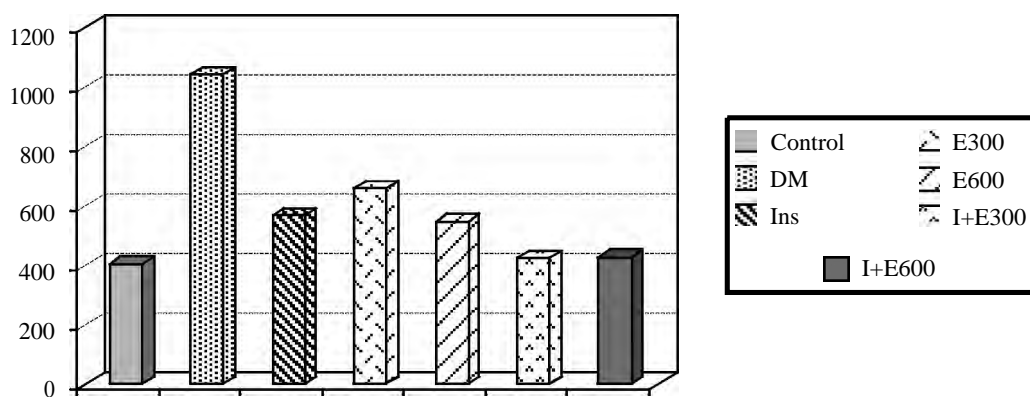
Group II (DM) : Diabetics, untreated.

Group V (E600) : Diabetics, receiving vitamin E injection (600mg/kg).

Group III (INS): Diabetics, treated with insulin only.

Group VI (I+E300) : Diabetics treated with insulin and vitamin E (300mg/kg).

Group VII (I+E600) : Diabetics treated with insulin and vitamin E (600mg/kg).

Fig. (1): Annexin V levels ($\mu\text{g}/\text{mg ptn}$) in the heart tissues of studied groups by RT-PCR (no=70).Table (3): Mean levels of iNOS ($\mu\text{g}/\text{mg ptn}$) and cGMP (n mol/mg ptn) in the heart tissues of studied groups.

Groups	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
No.	10	10	10	10	10	10	10
iNOS	169.1300± 28.67#	451.4± 106.132*	276.760± 63.695 *#	356.03± 77.572*	337.42± 85.82*#	231.360± 57.68#•@	231.650± 45.0868#•@
cGMP	1.252± 0.4#	3.57± 0.57*	2.6± 0.46*#	2.3± 0.82*#	2.04± 0.67*#	1.37± 0.39#Δ•	1.154± 0.314Δ#•@

* Significant with Group I.

Significant with Group II.

• Significant with Group IV.

@ Significant with Group V.

Δ Significant with Group III.

Group I (cont) : Controls.

Group IV (E300) : Diabetics, receiving 300mg/kg Vit. E only.

Group II (DM) : Diabetics, untreated.

Group V (E600) : Diabetics, receiving vitamin E injection (600mg/kg).

Group III (INS): Diabetics, treated with insulin only.

Group VI (I+E300) : Diabetics treated with insulin and vitamin E (300mg/kg).

Group VII (I+E600) : Diabetics treated with insulin and vitamin E (600mg/kg).

The effects of diabetes on the expression of the pro-inflammatory parameter, inducible nitric oxide synthase (iNOS) and the mediator of NO; cGMP are shown in Table (3).

There was a significant increase in both iNOS & cGMP in the hearts of untreated DM group ($p<0.001$). Treatment with insulin alone (Group III), or Vitamin E alone (groups IV and V) did not decrease iNOS levels to controls values. These were apparent in significantly higher levels of iNOS in groups III, IV, V when compared with controls ($p<0.05$), ($p<0.001$), ($p<0.001$) respective-

ly. A similar observation was obtained with cGMP levels when groups III, IV and V were compared with controls ($p<0.001$), ($p<0.05$), ($p<0.001$) respectively. It is apparent that both treatments decreased these two parameters significantly when compared with group II (untreated DM). The exception was levels of iNOS in group IV (low dose Vit E treatment) ($p=0.077$). Otherwise iNOS & cGMP were significantly decreased in group III compared with group II. Mean cGMP levels in group IV were significantly lower than those in group II ($p<0.001$) and iNOS & cGMP in group V were significantly less than in group II ($p<0.05$)

and ($p < 0.001$). In the groups receiving combined treatment with insulin and either doses of Vit E (groups VI and VII), levels were not significantly different from those of the control group. It was apparent that iNOS and cGMP levels in groups VI and VII were less than that in group IV ($p < 0.005$), ($p < 0.005$), ($p < 0.005$), ($p < 0.001$) respectively. Also they were significantly less in the last two groups than in group V ($p < 0.001$) for all parameters; except for cGMP in group VI ($p = 0.168$).

In addition, cGMP was significantly lower in group VI & group VII ($p < 0.001$ in both) as compared with group III.

These results indicate that both hyperglycemia and oxidative stress play a significant role in the inflammatory process in Type I diabetes. In addition, combined treatment with insulin and Vitamin E has a better response than a single treatment in fighting the inflammatory pathogenesis in this type of diabetes.

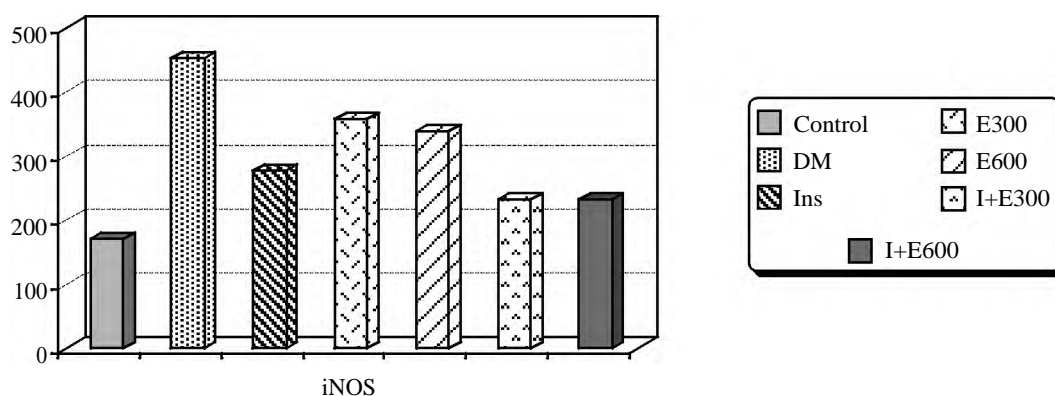


Fig. (2): Mean levels of iNOS ($\mu\text{g}/\text{mg ptn}$) in the heart tissues of studied groups (no=70).

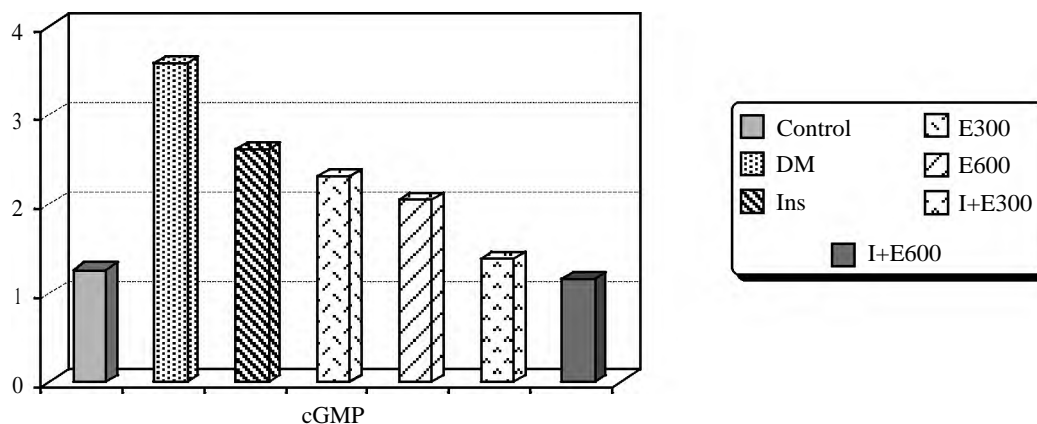


Fig. (3): Mean levels of cGMP (n mol/mg ptn) in the heart tissue of studied groups (no=70).

Table (4): Mean levels of cardiac enzymes AST, ALT & CPK (μmol) in the studied groups (no=70).

Groups	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
No.	10	10	10	10	10	10	10
AST	5.820± 1.766#	25.95± 8.13*	11.940± 2.4047*#	11.71± 2.17*#	10.52± 1.78#	7.66± 1.5086#	7.35± 1.596#
ALT	4.31± 0.702#	9.50± 2.160*	7.17± 1.493*#	8.23± 1.885*	6.85± 1.564*#	6.17± 1.125#	5.92± 1.357#•
CPK	2.86± 0.574#	6.00± 1.204*	3.35± 0.81#	2.80± 0.87#	2.32± 0.691#	1.95± 0.783#Δ	1.85± 0.737#Δ

* Significant with Group I.
@ Significant with Group V.
Group I (cont) : Controls.
Group II (DM) : Diabetics, untreated.
Group III (INS): Diabetics, treated with insulin only.

Significant with Group II.
Δ Significant with Group III.

• Significant with Group IV.

Group IV (E300) : Diabetics, receiving 300mg/kg Vit. E only.
Group V (E600) : Diabetics, receiving vitamin E injection (600mg/kg).
Group VI (I+E300) : Diabetics treated with insulin and vitamin E (300mg/kg).
Group VII (I+E600): Diabetics treated with insulin and vitamin E (600mg/kg).

The effects of diabetes on the laboratory indicators of cardiac injury are shown in Table (4). Cardiac enzymes were increased significantly in group II ($p<0.001$) than in control (group I). In groups treated with either vitamin E or insulin (groups III, IV and V) levels were significantly less than in group II, but were still higher than in the control group (except for CPK). However, treatment with either doses of vitamin E combined with insulin (groups VI & VII) decreased levels to the controls value.

The effect of diabetes and various types of treatment on oxidative markers MDA & the antioxidant GPX in the heart tissues of studied groups and their ratio GPX/MDA were shown in Table (5). MDA, the oxidative marker, was significantly

elevated ($p<0.01$) in group II as compared with controls. In insulin-only treated rats (group III) it was still higher than in controls, but it was less than that in group II ($p<0.01$). However, in other groups (IV, V, VI, VII) level did not differ from those in controls and were significantly less than group II.

On the other hand, levels of the antioxidant GPX, were significantly decreased in group II. In groups III, IV, V, VI, VII levels did not differ from those in controls.

Antioxidative status is better indicated by the ratio of GPX/MDA. This was also decreased in groups II and III. However, in groups IV, V, VI and VII levels were similar to those of control.

Table (5): Mean levels of oxidative markers MDA (n mol/mg ptn) and the antioxidant GPX ($\mu\text{mol}/\text{mg}$ tissue) in the heart tissues of studied groups and their ratio GPX/MDA.

Groups	Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
No.	10	10	10	10	10	10	10
MDA	0.107± 0.0235#	0.3440± 0.095*	0.2020± 0.055*#@•	0.112± 0.034#	0.109± 0.042#	0.109± 0.0398#Δ	0.084± 0.0171#Δ
GPX	1.986± 0.4993#	0.087± 0.015*	1.71± 0.407#	1.54± 0.509#	1.57± 0.39#	1.632± 0.45#	1.91± 0.46#
GPX/MDA	19.58± 6.7#	0.27± 0.08*	9.00± 2.97*	15.04± 7.1#	17.00± 8.3#	15.56± 3.2#	24.01± 9.25#Δ•

* Significant with Group I.

@ Significant with Group V.

Group I (cont) : Controls.

Group II (DM) : Diabetics, untreated.

Group III (INS): Diabetics, treated with insulin only.

Significant with Group II.

Δ Significant with Group III.

• Significant with Group IV.

Group IV (E300) : Diabetics, receiving 300mg/kg Vit. E only.

Group V (E600) : Diabetics, receiving vitamin E injection (600mg/kg).

Group VI (I+E300) : Diabetics treated with insulin and vitamin E (300mg/kg).

Group VII (I+E600) : Diabetics treated with insulin and vitamin E (600mg/kg).

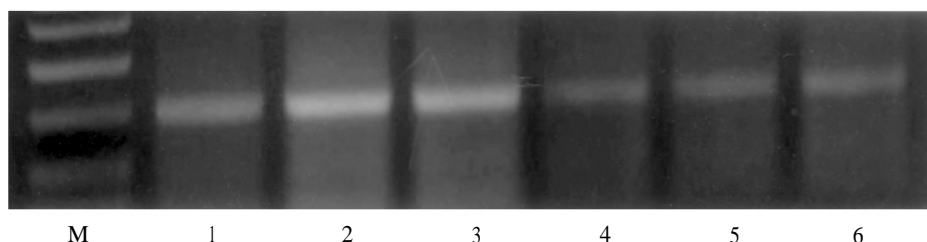


Fig. (4).

Agarose gel electrophoresis showing product of annexin gene expression:

Lane M: PCR marker.

Lane 1: Gene product in control group.

Lane 2&3: Gene product in diabetic group.

Lane 4: Gene product in diabetic group receiving insulin.

Lane 5: Gene product in diabetic group receiving vitamin E.

Lane 6: Gene product in diabetic group receiving insulin and vit E.

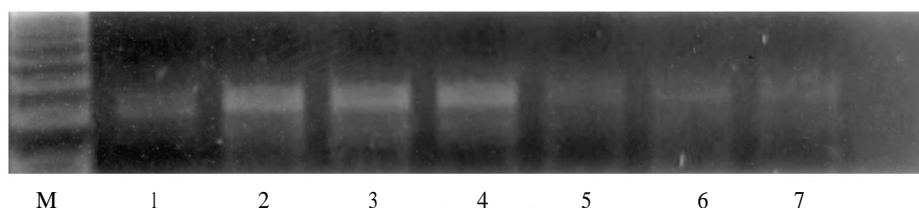


Fig. (5).

Agarose gel electrophoresis showing product of iNOS gene expression:

Lane M: PCR marker.

Lane 1: Gene product in control group.

Lane 2,3&4: Gene product in diabetic group.

Lane 5: Gene product in diabetic group receiving insulin.

Lane 6: Gene product in diabetic group receiving vitamin E.

Lane 7: Gene product in diabetic group receiving insulin & vit E.

Discussion

Recently it has been shown that annexin V is an apoptotic marker in the heart [1]. The aim of the present study was to explore whether in the heart annexin V content changes during diabetes, in order to use it as a prognostic factor in cardiac affection in DM. There is increased evidence that cell death after myocardial ischemia and reperfusion may begin as apoptosis rather than necrosis [15]. Furthermore, the study was done to show the effect of treatment with insulin and vitamin E (300mg & 600mg/Kg) either alone or in combination.

This is the first study we are aware of that has examined this relation combined with biochemical markers of cardiac injury and oxidative status.

There were three major new findings in the present study. First; annexin V levels were significantly elevated in the heart of diabetic rats compared with controls. According to Carmos et al. [2] who found that annexin V exhibits a proapoptotic effect in cardiomyocytes in ischemic and failing hearts, we can conclude that this change in annexin V content indicates that apoptosis is the basis of cardiac injury as confirmed by the elevated cardiac enzymes AST, ALT & CPK.

Second, we found that iNOS protein of the heart is elevated in diabetic rats. On the basis of these findings, we can suggest that inflammation could play a role in the pathogenesis of diabetic cardiomyopathy in type 1 diabetes. Fujimoto et al. [16] showed this relation in association with type 2 diabetes. Also, Cheng et al. [17] found that the activity of iNOS was three fold higher in the heart of diabetic rats than in controls. In addition, they found selective inhibition of iNOS restored cardiovascular responses to noradrenaline. Several investigators, have shown impairment of contractile

force generation, abnormal filling and delayed relaxation of cardiac muscle in diabetic patients and experimental settings [18,19,20]. Cheng et al. [17] revealed the contribution of iNOS in the depressed cardiovascular function at the acute phase of STZ-induced diabetes.

Third, the effect of various types of treatment of diabetes which were used here showed the beneficial effects of the use of both insulin and the antioxidant, vitamin E. These results are consistent with the degenerative role of hyperglycemia on cellular reducing equivalent homeostasis and antioxidant defense and provide further evidence that pharmacological intervention of different antioxidants may have significant implications in the prevention of the pro-oxidant features of diabetes. These results were supported by many further studies [11,21]. Furthermore, Bojunga et al. [22] showed that antioxidative treatment was capable of reversing changes in the NO-cGMP system and may therefore be an important option for preventing vascular damage in diabetes mellitus. In addition, Vierira de Costa & Vianna [23] found that vitamin E was able to modulate blood pressure and lipidimic profile as well. However, the best responses were obtained with combined treatment of insulin together with either doses of vitamin E. These results were in agreement with that of Yoshida et al. [24]. They found that combined treatment with vitamin E and insulin was useful in preventing the development and progression of diabetic cataract. However, Economides et al. [25] did not recommend the use of high doses of vitamin E in diabetic patients because of their worsening effects on endothelial or left ventricular functions.

Conclusion: These results suggest that annexin V can be used as a prognostic factor in cardiac affection in DM. In addition, they provide further evidence that pharmacological intervention by

different antioxidants may have a significant role in the prevention of the pro-oxidant features of diabetes.

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