Original Article

Protective Effects of Annona Squamosa on Cardiovascular Congenital Anomalies in Streptozotocin Induced Diabetic Albino Rats

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Abstract

Objective: To evaluate the protective effects of aqueous leave extract of *Annona squamosa* on cardio vascular congenital anomalies in diabetic albino rats.

Methods: It was a randomized control study, conducted in the Department of Anatomy, University of Health Sciences Lahore Pakistan, from May 2007 to 08. 18 adult albino pregnant rats with initial weight of 200 – 250 gm were randomly selected and divided into three groups; Group A served as control, whereas diabetes was induced by single injection of streptozotocin (45 mg / kg) on 8th gestational day in groups B and C. The diabetes was confirmed by checking blood glucose level from tail vein of the rats. Group C was additionally treated with the aqueous extract of *Annona squamosa* leaf (350 mg / kg / day) from 10th to 20th day of pregnancy. Fetuses were obtained by sacrificing the animals on 20th gestational day. Fetuses were euthanized, decapitated and fixed in 10% neutral buffered formalin; thoracic cavity was opened, heart and great vessels were observed thoroughly under stereomicroscope. Heart was removed and processed for histological examination; serial sections, 5 µm thick, were obtained, stained with H & E and examined with the light microscope. The design and protocol of the study was approved by the ethical committee of the institute.

Results: 18.2% fetuses from B group showed transposition of great vessels and atrial septal defect. Mean thickness of interventricular septum, in group B was significantly increased (881.0 ± 117.4 µ) as compared to that in groups A (487.7 ± 141.0 µ) and C (558.8 ± 86.4 µ, $p < 0.05$) indicating marked septal hypertrophy. Valvular defects were also observed in fetuses from group B. Such anomalies were not discernable in group C fetuses.

Conclusion: It is concluded that use of *Annona squamosa* leave extract alleviates diabetes induced cardiac malformations.

Key Words: Diabetes mellitus, *Annona squamosa*, cardiac anomalies.

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Introduction

Diabetes mellitus commonly complicates pregnancy[1]. It commonly affects cardiovascular, genitourinary musculoskeletal and central nervous systems[2]. Con-

genital heart defects are found to be the most frequent malformations amongst malformed infants of diabetic mothers. These congenital anomalies are 9.7 and 20.6 times more in pregestational and gestational type II diabetes respectively when compared with the non diabetics.[3] Pre gestational diabetes is documented to be strongly associated with defects of early cardiogenesis rather than shunting defects or cardimyopathies. Maternal diabetes is considered to be an independent risk factor for cardiovascular malformations because none of social or environmental variables confounded the association. This increased risk of cardiovascular malformations is potentially preventable by proper preconception and obstetric care.[4]

Dextrocardia (laterality defects), cardiac looping abnormalities (transposition of the great vessels), valvular and septal defects in offspring of diabetic mothers in population based studies are well documented.[4,5]

Several mechanisms operate in the pathogenesis of embryo – toxicity associated with diabetes; Hyperglycemia, enhanced activities of polyol pathways, defective reducing mechanisms and increased formation of reactive oxygen species leading to DNA damage, play a central role in the development of embryonic malformations associated with diabetes.[2,6]

In this perspective antioxidant treatment has largely been major focus of different workers to prevent diabetes associated congenital malformations[7]. Several antioxidants (vitamins; E,[8] C,[9] B_{12}[10] and alpha lipoic acid),[11] in different combinations,[12,13] doses[14] and routes,[8] have proven to be beneficial in preventing or at least decreasing the incidence of congenital anomalies associated with maternal diabetes.

Annona Squamosa is a tropical plant, of family Annonacae, commonly known as custard apple, sugar apple, sweet sop, sharifa etc, with a varied medicinal uses of all parts of the plant.[15] Its leaves along with black pepper grains are used extensively as a folk remedy of diabetes.[16] Ethanolic as well as aqueous extracts of *Annona squamosa* leaves have shown antidiabetic, hypoglycemic and antioxidant properties in diabetic experimental animals.[16,17] Leaves of *Annona squamosa* contain flavonoids, terpenoids and alkaloids responsible for its pharmacological activities.[15,16]

Although *Annona squamosa* leaf extract is documented to have anti diabetic, hypoglycemic and antioxidant properties in experimental animals, yet no study is available revealing its protective effects on cardiovascular congenital anomalies in diabetic rats.

This study was therefore planned to evaluate possible protection by *Annona squamosa* leaf extract against the teratogenic effects on developing heart in streptozotocin induced diabetic rats.

Materials and Methods

The study was conducted in the Department of Anatomy, University of Health Sciences Lahore, Pakistan over one year; May 2007 – 08. 24 adult albino rats (18 females and 6 males), weighing from 200 to 250 grams, procured from the National Institute of Health, Islamabad, were used in the study. The animals were housed in the experimental research laboratory of University of Health Sciences, Lahore (Pakistan), under controlled conditions of temperature (22 ± 0.5°C), humidity (50 ± 10%) and light and dark cycle of 12 hours; they were fed on rat chow and tap water ad labitum; the care and handling of the animals throughout the study followed the regulations set by the ethical committee of the institute.

After a period of 1 week of acclimatization, male and female rats were put together in a ratio of 1:3 in a single cage. Females were examined every morning for the presence of vaginal plug to determine the occurrence of pregnancy. Midnight of the night of mating was designated day 0 of embryonic development.

Pregnant rats were randomly divided into 3 groups A, B and C, each containing 6 animals and served as control, diabetic and treated groups respectively. Diabetes was induced on 8th gestational day, with a single intra peritoneal injection of 45 mg/kg of Streptozotocin (Sigma Chemical Co., St Louis, USA) dissolved in 1ml of citrate buffer (0.1 M, pH 4.5) in the overnight starved rats of groups B and C. The rats were allowed to drink 5% sucrose solution overnight after injection to overcome the drug-induced hypoglycemia. The rats of control group were injected with weight related quantity of solvent only.

Random blood glucose levels were checked on 10th gestational day by Clever Check glucometer (Germany Code # 1362) by taking a drop of blood from rat's tail vein. Blood glucose levels of 250 mg/dl and above in treated rats, were considered as diabetic. Normal blood glucose level of female rat is 163 – 174 mg/dl.

Fresh leaves of *Annona squamosa* were collected from the gardens in Malir, Sindh, Pakistan, in the month of April and May. Identification of the leaves was done by using standard botanical monographs. The aqueous extract of the leaves was prepared by cold

maceration of 50 g of the shade dried leaf powder in 150 ml of distilled water, allowed to stand overnight, and boiled till the volume was reduced to half. The solution was then cooled, filtered by using fine muslin mesh. The filtrate was concentrated in rotary vacuum evaporator (Heidolph) at 70°C, 170 rpm and dried in freeze drier (Heidolph) at PCSIR laboratories Lahore, Pakistan. The residue was stored in a refrigerator at 2 – 8°C for subsequent experiments.

Diabetic rats of group C were orally given *Annona squamosa* leave extract, from 10th to 20th gestational days, in three divided doses dissolved in distilled water so that the total dose was 350 mg / kg / day. Blood glucose levels were measured and recorded on 10th gestational day and then on alternate days throughout the gestation.

Live fetuses were delivered on the 20th gestational day by caesarian section, euthanized by immersing them in cold physiological saline solution. They were later decapitated and fixed in 10% neutral formalin for 72 hours.

The thoracic cavity of the fetuses was opened up and the principle vessels and heart were identified and observed under dissecting microscope for any discernable malformation.

5 µm thick serial sections of fetal heart were obtained in a usual way and stained with haematoxylin and eosin technique for histological examination with the light microscope.

Statistical Analysis

The data was entered and analyzed using SPSS (Statistical Package for Social Sciences) version 15.0. One way ANOVA was applied to observe group mean differences and Post Hoc Tukey test was applied to observe which mean differs. Sphapiro – Wilk test was applied to check normality. Pearson Chi square and Fisher exact test was applied to observe associations between qualitative variables. A p-value of < 0.05 was considered statistically significant.

Results

The origin of aorta and pulmonary trunk was found to be normal in the fetuses from groups A and C (Fig. 1a). Transposition of great vessels was observed in 18.2% fetuses in the group B (Fig. 1b).

Marked septal hypertrophy of the interventricular septum in the group B was observed (Fig. 2, B_1, B_2, B_3); ANOVA showed significant difference in the thickness of inter-ventricular septum of the three groups. Tukey HSD test showed that this difference between the groups A&B and B&C was statistically significant. The difference between the groups A&C was, however statistically insignificant (Table 1).

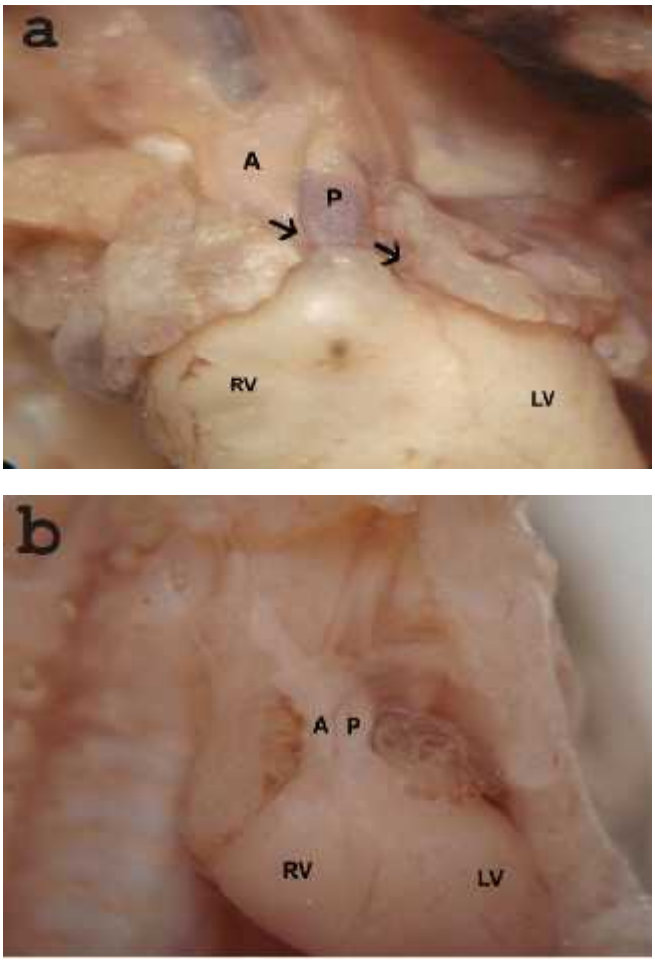

Fig. 1: Photographs of fetal heart from control group (a) showing normal origins of aorta (A) from left ventricle (LV) and pulmonary trunk (P) from right ventricle (RV). In diabetic group (b) the aorta (A) is arising from right ventricle (RV) and pulmonary trunk (P) from left ventricle (LV).

Inter atrial septum was normal and complete, dividing the two atrial chambers completely in the fetuses of groups A and C, 18.2% fetuses from the group B had atrial septal defect (Fig. 2, B_3).

Cusps of some of AV valves from the group B were not comparable to those from groups A&C (Fig. 3B). Accumulation of basophilic tissue (Fig. 3 C) was more evident at the bases of other semilunar valves; the material was composed of cluster of darkly stain-

Table 1: Comparison of the thickness of inter-ventricular septum among the 3 groups.

Comparison Among Groups		Mean Difference (α – β)	Level of Significance P-Value
Group (α)	Group compared (β)		
A	B	-393.3	< 0.001*
	C	-71.1	0.131
B	A	393.3	< 0.001*
	C	322.2	< 0.001*
C	A	71.1	0.131
	B	-322.2	< 0.001*

* p value< 0.05

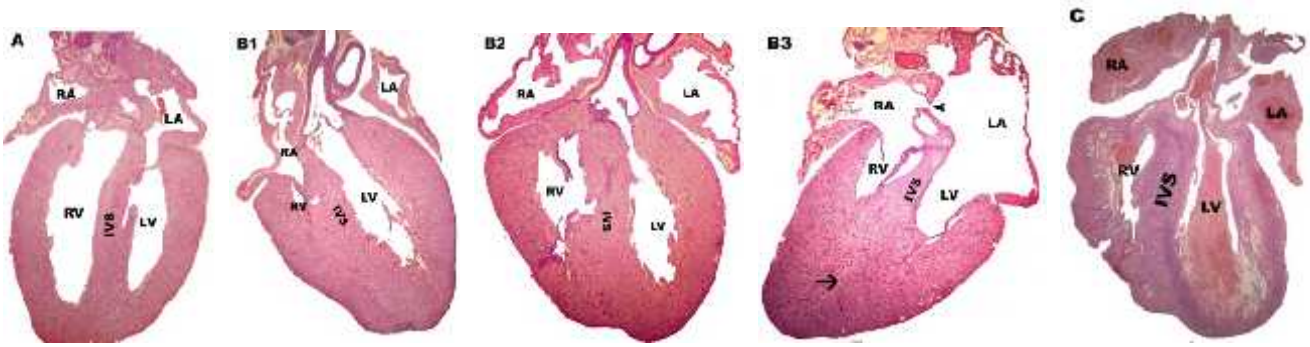

Fig. 2: Photomicrographs of fetal hearts from control (A), and treated groups (C), showing right and left atrial cavities (RA and LA), right and left ventricular cavities (RV and LV) separated by inter ventricular septum (IVS), increased thickness of IVS, myocardial hypertrophy (arrow in B_3) and atrial septal defect (arrow head in B_3) in fetal hearts from diabetic group (B_1, B_2, B_3). H&E stain × 40.

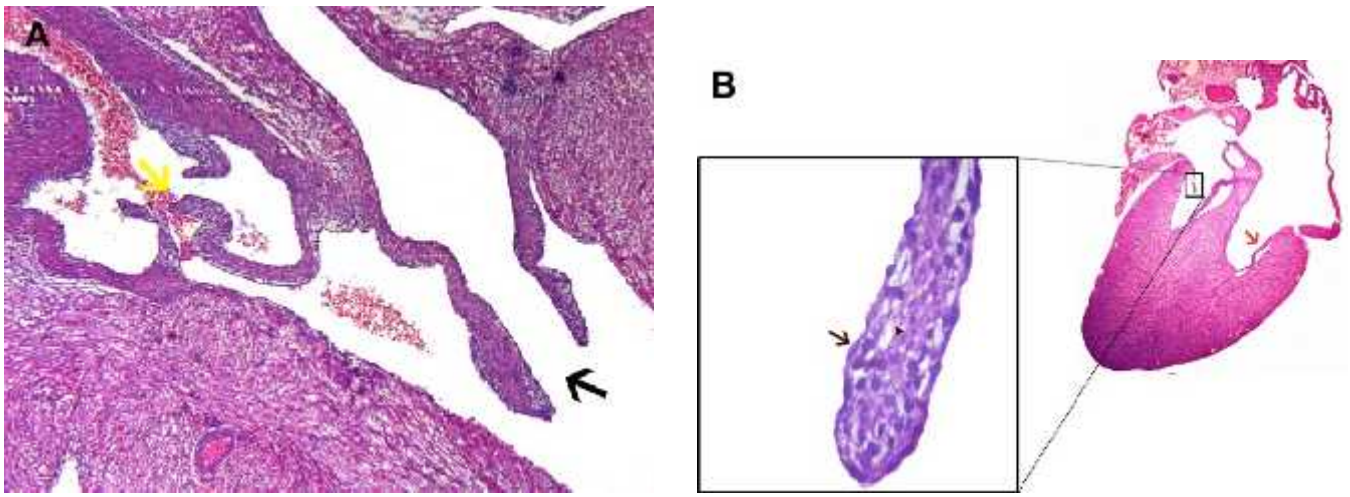

Fig. 3a, b: Photomicrographs showing normal semilunar (yellow arrow) and AV valves (black arrow) of fetal heart from control group (A), malformed AV valve (red arrow in Fig. B) from diabetic group; inset showing connective tissue core covered by endocardial cells (arrow head and arrow in Fig. B respectively). Accumulation of basophilic material

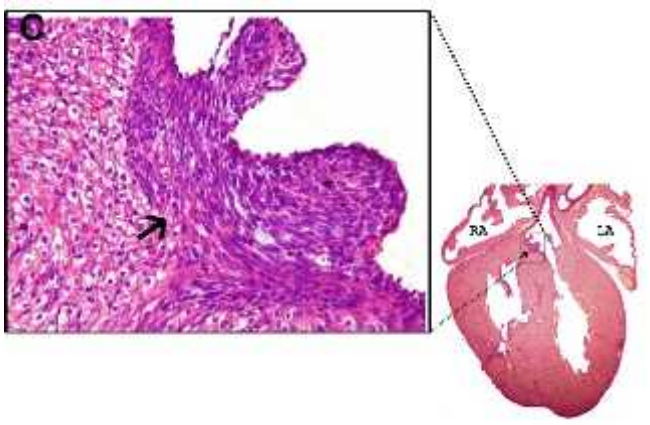

Fig. 3c: (arrow in Fig. C) is seen at the base of semilunar valve. H&E stain × 40 and 100.

ing spindle shaped cells with central elongated nuclei. This feature was not discernable in valves from animals of both groups A and C.

Discussion

Cardiovascular anomaly like transposition of great vessels (Fig. 1b) was observed in the fetuses of diabetic mothers. It has been widely reported before.[2,4,5]

Maternal diabetes resulting in altered expression of BMP – 4 (bone morphogenic protein 4) and PAX – 3 (a paired box gene), is documented to be the underlying cause of abnormalities of the outflow tract of fetal heart. These genes are important regulators for induction, maintenance, migration, differentiation and survival of the cardiac neural crest cells, contributing to conotruncal septation of fetal heart.[18]

Such abnormalities of vessels were not observed in the fetuses of the group treated by aqueous leave extract of *Annona squamosa*. It suggests that markedly elevated level of glucose is involved in the pathogenesis of these vascular abnormalities by inhibiting the migration of cardiac neural crest cells, as is reported earlier.[18] *Annona squamosa* presumably exerted its protective role by controlling the hyperglycemia.

Thickness of the interventricular septum was also increased significantly in the fetuses of diabetic group as compared to the controls (Fig. 2 B_1, B_2 and B_3); this has been reported earlier invariably both in human[19] and animal models.[20]

Maternal hyperglycemia leads to fetal hyperglycemia that stimulates fetal pancreas and results in fetal hyperinsulinemia. Increased insulin level leads to increased glycogenesis, lipogenesis and protein synthesis in fetal organs; this is proposed to be conducive for hypertrophic cardiomyopathy and septal hypertrophy.[19]

Recently molecular basis of the diabetic cardiomyopathy have been addressed. Down regulation of the gene and mRNA for GLUT – 1 (glucose transporter, basal form) and GLUT – 4 (insulin responsive form), on myocardial cells of the fetus in maternal diabetes has been reported. Rate limiting enzyme in glucose metabolism is also found to be inhibited, suggesting decreased entry and utilization of glucose despite hyperglycaemia. There is associated up regulation of genes involved in cellular fatty acid uptake by diabetic myocardium. Altered expression of genes involved in glutathione (natural cellular antioxidant defense mechanism) metabolism is also observed; this suggests role of oxidative stress in the pathogenesis of diabetic cardiomyopathy.[21]

The hypertrophy was not discernable in the group treated with *Annona squamosa*, suggesting the protection afforded by its antioxidant property.

Atrioventricular valves in few fetuses of diabetic group appeared malformed (Fig. 3, B) and the accumulation of basophilic material was observed at the basis of valves (Fig. 3, C). This could be mesenchyme responsible for connective tissue deposition and transforming into fibroblasts for maturation of valves. This could possibly also indicate the accumulation of cardiac neural crest cells.[22] Both suggest immaturity in diabetic group. Hyperglycemia is documented to reduce the expression of VEGF which consequently inhibits the epithelial mesenchymal transformation, necessary for normal development of cardiac valves. It results in developmental abnormalities of heart valves associated with maternal diabetes.[23]

Septal defect involving atria was also observed in the diabetic group (Fig. 2, B_3); this is quite commonly reported to be associated with the diabetic pregnancy.[2,5] Abnormal expression of genes involved in the regulation of early cardiogenesis is the probable responsible factor.[18,22]

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Conclusion

Present study, probably, for the first time reported that *Annona squamosa* leave extract protects against the cardiovascular congenital anomalies associated with maternal diabetes in albino rats. It needs further verification and confirmation by continued work in humans to evaluate the effect of the leave extract of *Annona squamosa*. Further, its proper chemical analysis needs to be undertaken to identify antidiabetic and cardiovascular protective components.

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