ORIGINAL ARTICLE

EFFECTS OF ANNONA SQUAMOSA LEAVE EXTRACT ON GROSS CONGENITAL ANOMALIES IN DIABETIC ALBINO RATS

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ABSTRACT

Objective: To investigate the effects of aqueous leave extract of Annona squamosa on gross congenital anomalies in diabetic albino rats.

Study Design: A randomized controlled trail (RCT) study.

Place and duration of study: This study was conducted during the period of one year in the Anatomy department of University of Health Sciences (UHS) Lahore, Pakistan.

Materials and Methods: Eighteen albino pregnant rats were randomly divided into three equal groups; Control (A), Diabetic (B) and Treated (C). Diabetes was induced by single intra peritoneal injection of streptozocin (STZ, 45 mg/kg), in the rats of groups B and C on 8th gestational day. Group C rats were additionally given the Annona squamosa leave extract (350mg/kg/day) throughout the gestation after the confirmation of diabetes by checking blood glucose levels on 10th gestational day. Fetuses were obtained by sacrificing the animals on 20th gestational day; these were euthanized; their birth weight and crown rump length (CRL) were recorded and gross malformations were observed under stereomicroscope. After fixation with 10% formalin, the position of thoracic and abdominal viscera was also observed.

Results: Mean birth weight and CRL in group B fetuses were significantly low (3.2±0.9g & 3.3±0.5cm) as compared to group A (4.7±0.5g & 4.3±0.3cm) and group C (3.8±0.2g & 3.9±0.2cm, $p<0.05$). Group B fetuses showed high frequency of craniofacial malformations and positional abnormalities of heart, diaphragm and kidneys. The group C fetuses did not show such malformations.

Conclusion: It is concluded that gestational use of Annona squamosa tends to alleviate the diabetes induced fetal malformations.

Keywords: Diabetes mellitus, Congenital anomalies, Annona squamosa extract

INTRODUCTION

Diabetes mellitus commonly complicates pregnancy[1]; it is associated with 2-8 fold increase in the rate of major fetal anomalies[2]. Adverse effects of maternal diabetes on newborn depend upon its severity[3]. Poor periconceptional maternal glycemic control may be associated with a birth defect rate as high as 20%[4]. Diabetes generally affects almost every developing major organ system of body, including central nervous, cardiovascular, genitourinary and musculoskeletal[5]. Craniofacial abnormalities include micrognathia, exencephaly, short nose, distorded midface, hydrocephaly, meningocele, oblique facial clefts and low set ears[6,7].

Reported positional abnormalities of viscera include dextrocardia, cardiac looping abnormalities and situs inversus[8].

Hyperglycemia induces an increased metabolic overload on mitochondria, leading to increased formation of reactive oxygen species, this oxidative stress is augmented by increased lipid peroxidation coupled with defective antioxidant mechanisms[9]. An excess of reactive oxygen species leads to DNA damage through the production of superoxide and hydrogen peroxide radicals that play a central role in the development of embryonic malformations in diabetic conditions[10], both in vivo and in vitro [9].

In this perspective antioxidant treatment has largely been major focus of different workers to prevent diabetes associated congenital malformations[11]. Several antioxidants (vitamins; E[12], C[13], B12[14] and alpha lipoic acid[6]), in different combinations[15,16], doses and routes[17], have proven to be beneficial in preventing or at least decreasing the incidence of congenital anomalies associated with maternal diabetes.

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Received: 13 March 2009; Accepted: 14 Oct 2009

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[18]. Its leaves with black pepper grains are used extensively as a folk remedy of diabetes[19]. Leaves of Annona squamosa contain flavonoids, terpenoids and alkaloids responsible for its pharmacological activities[18]. Ethanolic as well as aqueous extracts of Annona squamosa leaves had been reported to show antidiabetic, hypoglycemic and antioxidant properties in diabetic experimental animals[19-21], however, there is hardly any study on its protective effect on congenital anomalies in diabetic rats; this study was, therefore, planned to investigate the possible protection afforded by Annona squamosa leave extract against diabetes induced congenital anomalies in albino rats.

MATERIALS AND METHODS

These randomized controlled trails (RCT) was carried out in the department of University of Health Sciences (UHS) Lahore. Twenty four albino adult 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 UHS, Lahore, 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 libitum; the care and handling of the animals throughout the study followed the regulations set by the ethical committee of the University of Health Sciences.

After a period of 1 week of acclamatization, male and female rats were put together in a ratio of 1:3 in a single cage. Females were examined daily for vaginal plug. Night of mating was designated day 0 of embryonic development.

The pregnant rats were randomly divided into three 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 body weight of STZ (Sigma Chemical Co., St Louis, USA) dissolved in citrate buffer (0.1 M, pH 4.5) to the rats of groups B and C; these animals were kept starved overnight before injection. The rats were allowed to drink 5% sucrose solution overnight after injection to overcome the drug-induced hypoglycemia. The rats of group A were injected with comparable weight related quantity of solvent only.

Diabetes was confirmed on 10th gestational day; a drop of blood from rat's tail vein was used for checking random blood glucose level by Clever Check glucometer (Germany Code# 1362). Blood glucose levels of 250 mg/dl and above were considered as diabetic. Normal blood glucose level of female rat is 163-174 mg/dl.

Fresh leaves of Annona squamosa were collected during April/May from Malir, Sindh, Pakistan. 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 daily dissolved in distilled water so that the total dose was 350 mg/kg/day. Blood glucose levels were 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. Litter size, fetal birth weight and CRL were recorded and all the fetuses were examined for gross congenital anomalies, under a dissecting stereo microscope. Fetuses were euthanized by immersing them in cold saline; they were later decapitated and fixed in 10% neutral buffered formalin for 72 hours. The thoracic and abdominal cavities of the fetuses were opened

and observed under dissecting microscope for any discernable malformation. Position of thoracic and abdominal viscera was also observed.

Statistical Analysis:

The data was entered and analyzed using SPSS (Statistical Package for Social Sciences) version 15.0. Mean±S.D was given for quantitative variables. One way ANOVA (analysis of variance) was applied to compare means of different groups for quantitative variables followed by Post Hoc Tukey test for pair wise comparison. Pearson Chi square and Fisher exact tests were applied to observe associations between qualitative variables. A p-value of <0.05 was considered as statistically significant.

RESULTS

Total number of litters of group A was 37 and those in groups B and C were 22 and 17 respectively. ANOVA showed a significant difference in the fetal birth weight and CRL in all the groups, treatment with Annona squamosa tended to normalize it partially but the difference between the two groups (A&C), however, was statistically significant (Fig 1& 2).

The skin of control and treated fetuses was thick, healthy and pink in color; it was thin translucent and more vascular in 15 out of 22 (68.2%) fetuses of B group. Twelve (54.5%) fetuses from B group had rounded orbits as compared to elliptical outline in those from groups A and C (Fig. 3). Five (22.7%) fetuses of group B also showed notched auricle. Other facial features were well formed in all groups. Chi square test showed significant association between the occurrence of thin skin and rounded orbits of the fetuses with maternal diabetes (Table).

Ten fetuses (45.5%) from group B had abnormal position of heart, diaphragm (Fig. 4) and kidney (Fig. 5): including left deviation of heart, where right lung had covered most of the anterior surface of heart (Gig. 4B1) and obliquely placed diaphragm (Fig. 4B2). The position of these viscera were comparable in the fetuses from groups A & C.

DISCUSSION

Our observations showed that the maternal diabetes affected developing fetuses adversely. Findings, like decreased fetal birth weight and CRL are in line with those of the previous workers[12,22]. Diabetes produces these effects on

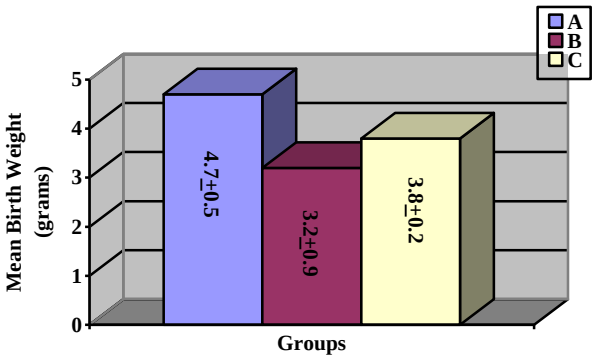

Fig. 1: Diagram showing the comparison of mean fetal birth weight in the three groups

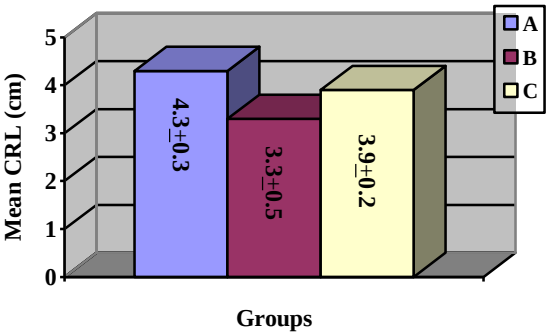

Fig. 2: Diagram showing the comparison of mean CRL of the fetuses from the three groups

Table: Distribution and analysis of abnormalities of skin and orbit in the three groups.

Groups	Skin		Total	Orbit		Total
	Thick	Thin		Elliptical	Rounded	
A	37	0	37	37	0	37
B	7	15	22	10	12	22
C	17	0	17	17	0	17
Chi-square Value=47.872 p- Value=<0.001			Chi-square Value=34.977 p- Value=<0.001			

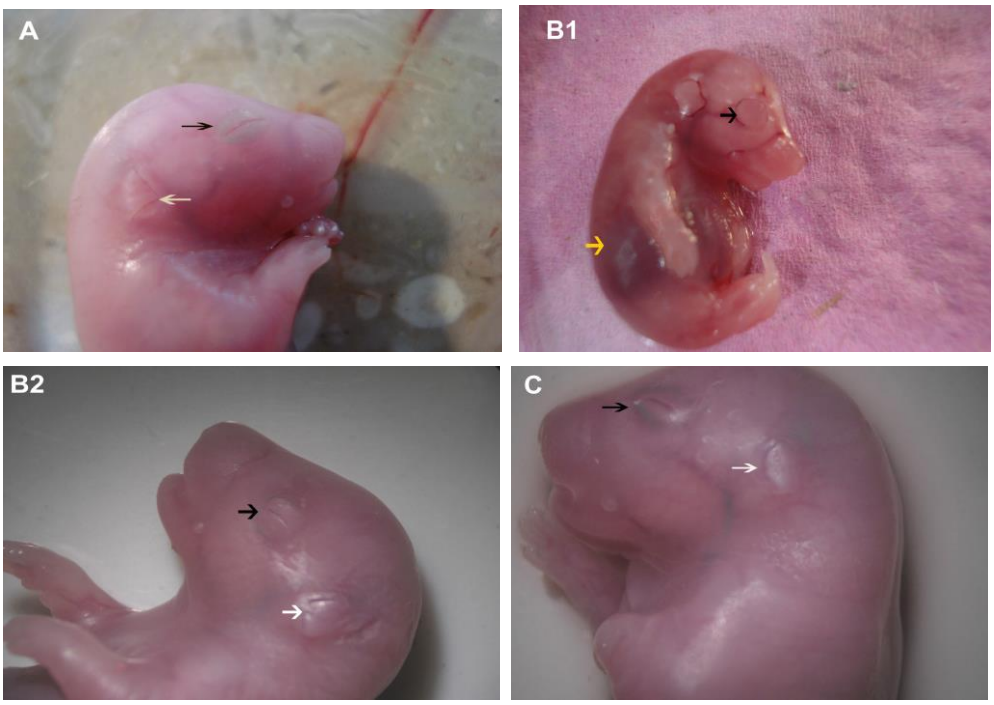

Fig. 3: Photograph showing elliptical orbit (black arrows) and normal auricle (white arrows) in groups A and C fetuses, rounded orbit (black arrows B1 and B2), auricular notch (white arrow B2) and thin, vascular skin (yellow arrow B1) in group B fetuses.

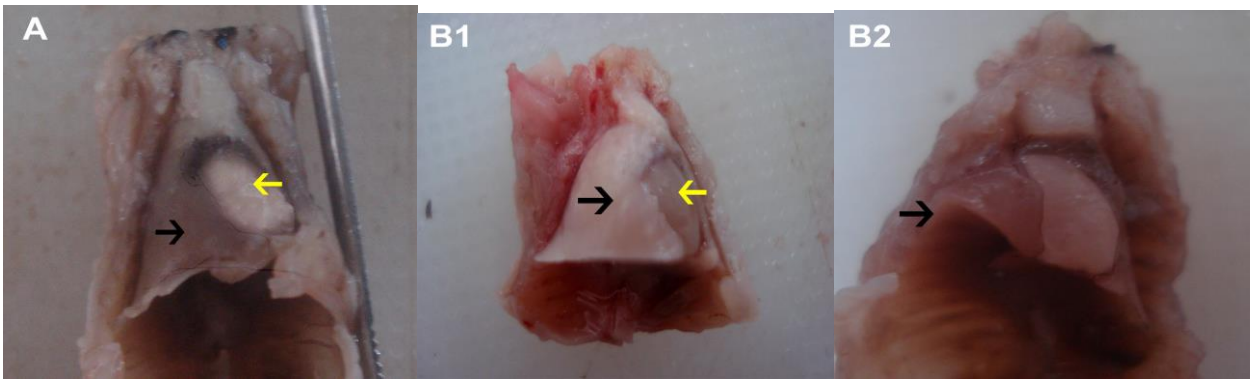

Fig. 4: Photograph showing normally placed heart (yellow arrow) and lungs (black arrow) in group A fetus, complete deviation of heart from midline position to the left (yellow arrow in B1) covered by right lung (black arrow) and obliquely placed diaphragm (black arrow in B2) from group B fetuses.

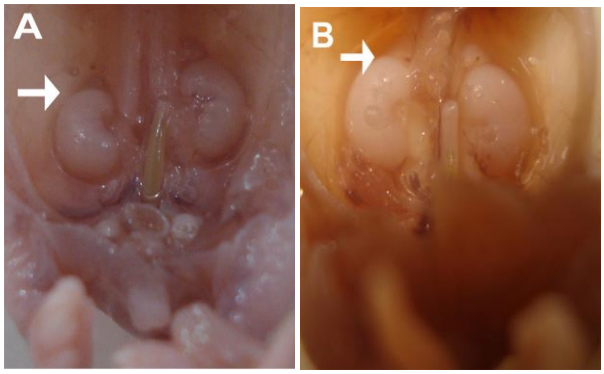

Fig. 5: Photograph showing normal position of group A fetal kidneys; right being lower than left (arrow in A) and superiorly displaced fetal kidney of group B (arrow in B).

account of placental insufficiency, its vasculopathy and impaired transport of amino acids[23].

In the current study Annona squamosa led to the improvement in the fetal birth weight and CRL. There is hardly any report available on the effects of Annona squamosa leave extract or any other part of the plant on fetal birth weight or CRL in any experimental model of study. However, treatment with vitamin E (an antioxidant) partially corrected the decreased fetal birth weight associated with maternal diabetes in experimental animals[22]. It could be implied from these reports that oxidative stress

in diabetes mellitus was responsible for congenital defects and this seems to have been neutralized by antioxidant treatment; there are, however, other unknown mechanisms involved in the process apart from oxidative stress.

Treatment with different doses[17] of vitamin E and C separately at different gestational periods[24] showed an improvement in the fetal CRL; however, a combined use of the two vitamins, E and C, in moderate or high doses did not produce synergistic effect[15].

Fetuses from diabetic group showed high frequency of craniofacial malformations, as was reported earlier[6,13]. Craniofacial structures are largely derived from neural crest cells, these cells are reported to be particularly poor in their antioxidant contents and therefore remain quite vulnerable to oxidative stress[25]. Cranial neural crest cell cultures had shown a reduced migrating capacity when exposed to diabetic environment in-vitro. The migration capacity of the neural crest cells can be normalized with the addition of antioxidants to the medium[25].

None of such malformation was discernable in the fetuses of the group treated with Annona squamosa leave extract. The extract might have exerted this protective effect, through its anti oxidant properties by normalizing the anti oxidant content of the vulnerable population of neural crest cells.

Maternal diabetes is known to be related to situs inversus and laterality sequences[8]. Present finding of abnormally placed heart and diaphragm might be explained as a part of such reported abnormalities of position of viscera in the fetuses of diabetic mothers.

Position of heart, diaphragm and other viscera was found to be normal and comparable in group A and C. This suggested that anti diabetic and antioxidant properties of aqueous extract of Annona squamosa leaves are responsible to correct the effects of oxidative stress in maternal diabetes.

CONCLUSION

Present study suggests that Annona squamosa leave extract upto some extent protects the gross congenital anomalies associated with maternal diabetes, in albino rats. Further work is needed to evaluate the effects of the leave extract of Annona squamosa in humans.

ACKNOWLEDGEMENTS

We are greatly indebted to Dr. Asmatullah, Associate Professor, Zoology Department, University of Punjab, Lahore, for his guidance and support. We are thankful to Dr. Amanullah for sharing his valuable knowledge in the statistical analysis of our work.

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